The Development of Inhibitory Mechanisms in Spinal Pain Pathways

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Abstract

The aim of this thesis was to investigate the postnatal development of inhibitory signalling in spinal pain pathways by the inhibitory transmitters GABA and glycine. Immunohistochemical studies and western blot analysis have shown that two key molecules, the scaffolding protein gephyrin and the potassium cation co-transporter KCC2, are postnatally upregulated in the rat superficial dorsal horn. Low levels of gephyrin limit postsynaptic clustering of $GABA_A$ and glycine receptors reducing the efficiency of synaptic inhibition, whereas the relative paucity of the cation-chloride co-transporter KCC2 in the neonatal period reduces the cell's ability to actively extrude chloride, producing a reversal of the electrochemical gradient. The upregulation of KCC2 was accelerated *in vivo* by peripheral inflammation and in cultured neurons by potassium induced depolarisation, while spike blockade with TTX produced the opposite effect. Gephyrin expression on the other hand, was not activity dependent.

The functional consequences of these low levels of KCC2 in the neonatal period upon GABA and glycine signalling were tested using intrathecal injections of GABA and glycine antagonists. In adults these antagonists are known to produce sensitisation of mechanical withdrawal thresholds but in neonates they had the opposite effect, causing reduced sensitivity. Correspondingly, GABA and glycine, whilst having no effect in adult rats produced sensitisation in neonatal rat pups. This difference was abolished on spinalisation, demonstrating the importance of supra-spinal connections in controlling segmental inhibition.

Benzodiazepines, are GABA agonists commonly used in neonatal intensive care. In order to test whether the results of this thesis have practical implications for clinical care, a questionnaire survey of neonatologists was carried out. Myoclonus and seizures were reported following the use of midazolam in young patients suggesting that low levels of KCC2 may also be a feature of the human neonatal CNS.

(290 words)

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List of Abbreviations

AMPA	Amino-hydroxy-methyl-isoxalone propionic acid
ANOVA	Analysis of variance
BDNF	Brain derived neurotrophic factor
BME	Basal medium eagle
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CSF	Cerebrospinal fluid
CWR	Cutaneous withdrawal reflex
D-APV	D-(-)-2-amino-5-phosphonovalerate
DIOA	[(dihydroindenyl)oxy] alkanoic acid
DIV	Day in vitro
DLF	Dorsolateral funiculus
DNIC	Diffuse noxious inhibitory control
DRG	Dorsal root ganglion
E	Embryonic day
FITC	Flurosceine avidin C
GABA	γ amino butyric acid
GABARAP	GABA receptor associated protein
GAD	Glutamic acid decarboxylase
GAPDH	Glyceralderhyde-3-phosphate dehydrogenase
GAT1	GABA transporter 1
GDNF	Glial cell derived neurotrophic factor
5-HT	5 hydroxy tryptamine
HRP	Horse radish peroxidase
IB4	Isolectin B4
IT	Intrathecal
KCC2	Potassium chloride cation co-transporter
LSN	Lateral spinal nucleus
LSO	Lateral superior olive
MAP2	Microtubule associated protein 2
mRNA	Messenger ribonucleic acid
NBQX	Tetrahydro-6-nitro-2,3-dioxo-benzoquinoxaline-7-sulfonamide
NeuN	Neuronal neuclear protein
NK1	Neurokinin 1
NGF	Nerve growth factor
NKCC1	Sodium potassium chloride cation co-transporter
NMDA	N-methyl-D-aspartate
P	Postnatal day
PAG	Periaqueductal grey matter
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCA	Postconceptional age

PKC	Calcium/diacyl-glycerol-dependent protein kinase
RVM	Rostroventral medulla
SLC12	Electroneutral cation-chloride coupled co-transporter gene family
s/mEPSC	Spontaneous/miniature excitatory postsynaptic current
s/mIPSC	Spontaneous/miniature inhibitory postsynaptic current
SG	Substantia gelatinosa
TrkA	Tyrosine receptor kinase A
TSA	Tyramide signal amplification
TTBS	Tris triton buffered saline
TTX	Tetrodotoxin
vFh	von Frey hair
VIAAT	Vesicular inhibitory amino acid transporter
WDR	Wide dynamic range

Acknowledgements

I would like to thank my supervisor, Professor Maria Fitzgerald for her patient support and guidance during the time that I was fortunate enough to work in her laboratory. I have been really touched by the help and support of all the scientists working both in her lab and elsewhere within the London Pain Consortium, without whom much of the work presented would not have been possible. I am particularly grateful to Dr Andrew Moss for teaching me a lot about western blot analysis and so much more about home improvements; to Jacqueta Meredith-Middleton for her expert assistance in the tissue culture lab (and everywhere else for that matter!); to Dr Sarah Slack for sharing her painstakingly acquired expertise for growing dorsal horn neurons in culture; to Dr Gareth Hathaway for his much valued assistance during my *in vivo* experiments, to Sharon Man for her help with those fiddly fluorogold injections, and to Dr Mark Baccei for contributing his words of wisdom during the preparation of this thesis. I greatly enjoyed working with Leda Lignos during her BSc project and took inspiration from her enthusiasm. The staff of the UCL post-room also assisted me with the postal questionnaire way beyond the call of duty. I would like to thank the Medical Research Council for giving me the chance to broaden my horizons, something that I truly believe will make me a more discerning doctor. Lastly my gratitude goes wholeheartedly to Ade & Maisie and to my friends & family for seeing me through the inevitable highs and lows of my time in research.

Chapter One

Introduction

1.1 Introduction

Mankind has pondered the mechanisms by which we feel pain since the 17th Century, when Descartes sought to understand how the heat of a fire caused a change in the skin which in turn lead to an unpleasant higher sensation .Yet as recently as 20 years ago, many paediatricians still believed that newborn infants could not feel pain. Ironically, over three hundred years earlier in his text 'The Children's Book', the physician Felix Wurtz, showed great insight when he wrote:

> *If new skin in old people be tender, what is it you think in a new born babe? Doth a small thing pain you so much on a finger, how painful is it then to a child, which is tormented all the body over, which hath but a tender new grown flesh? If such a perfect child is tormented so soon, what shall we think of a child, which stayed not in the wombe its full time? Surely it is twice worse with him? (Wurtz, 1656).*

The International Association for the Study of Pain (IASP) defines pain as '*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*'. This simple description is elegantly inclusive of the various types of pain experienced by human adults. However, it also highlights some of the problems associated with the recognition of neonatal pain. Firstly, the emotional response may be quantitatively or qualitatively

different in the youngest infants (Stevens et al, 1994), and secondly the very assumption that the patient can describe their pain is unrealistic in this age group. Thus the definition was further clarified in 2001 when the statement, '*the inability to communicate in no way negates the possibility that an individual is experiencing pain and is in need of appropriate pain-relieving treatment*', was added to draw attention to the issue of pain in vulnerable groups including pre-verbal children. The true significance of early sensory input has come to light more recently, with evidence emerging to suggest that our sensory input during infancy is likely to shape the way we feel pain for a lifetime (Anand, 2000). This thesis aims to contribute to the understanding of the developmental neurobiology of pain, by investigating the mechanisms involved in the development of inhibitory systems in spinal pain pathways.

Many infants are exposed to considerable pain as a result of disease processes, surgery or intensive care procedures, and research has shown that even the youngest infants respond to such painful stimulation (Fitzgerald, 1999). The adverse effects of this pain are not only immediate, but are also likely to have a longer-term impact on future sensation and behaviour (Porter et al, 1999). Despite this too few paediatric patients receive adequate analgesia, partly due to a lack of understanding of the fundamental mechanisms underlying sensory processing in infants. Mature sensory systems involved in pain processing are modulated both at the segmental level and by pathways providing descending inhibition. Therefore, under normal circumstances when a noxious stimulus causes excitation the magnitude and duration of this can be regulated. The exact nature of this modulation in the neonate remain poorly understood; and their relative lack of 'endogenous analgesia' leaves them

poorly able to 'dampen' noxious input to the central nervous system (CNS). The presence of inflammation or nerve damage can lead to central sensitisation and 'un-dampened' hypersensitive behaviour in adult animals, similar to that of the newborn (Woolf, 1994), thus the study of neonatal pain can also give important clues regarding the mechanism of pathological pain states in the mature nervous system.

Previous studies of spinal pain processing in both human infants and rat pups have demonstrated this relative lack of inhibitory control (Fitzgerald, 1999). Noxious stimulation in infants causes cruder 'whole body movements' which later mature into individual responses localised to an arm or leg (Fitzgerald et al, 1988). In rat pups, sensitivity to formalin injection is 10 fold greater, and the classical biphasic response is not seen before postnatal day 25 (P25) (Teng et al, 1998). While there are a number of possible mechanisms underlying the lack of inhibition in the post natal period, this thesis focuses upon the functional development of the two major inhibitory neurotransmitters GABA and glycine, and their receptors. In the adult spinal cord, GABA and glycine are the main inhibitory amino acid transmitters producing membrane hyperpolarisation through post-synaptic $GABA_A$, $GABA_B$, and glycine receptors and, depression of transmitter release through pre-synaptic $GABA_B$ receptors, providing a crucial role in limiting the spread of excitatory glutamatergic activity (Kerchner et al, 2001). Afferent evoked inhibitory post-synaptic potentials (IPSP) generally develop after excitatory postsynaptic potentials (EPSP) in the CNS, not because of absence of GABA or glycine terminals but because their functional properties are immature (Sanes et al, 2000). These differences may actually give them a critical role in developmental plasticity.

1.2 The Development of Pain Behaviour

Reflex responses to somatic stimuli begin as early as 7.5 weeks gestation in the human foetus (Bradley & Mistretta, 1975) and at day 15 in utero (E15) in the rat foetus, where the gestation period is 21.5 days (Narayanan et al, 1971). The perioral region is the first to become sensitive, with cephalo-caudal spread to the himdlimbs by 14 weeks in the human and 17 days in the rat. These early reflexes are not necessarily nociceptive, being mediated by large diameter A fibres. Indeed smaller diameter nerve fibres important for nociception (C fibres) only enter the human foetal spinal cord at 19 weeks in-utero (Konstantinidou et al, 1995). Early input appears to be important in the maturation of nociceptive responses, with premature infants having quite different responses to foetuses remaining in-utero at the same gestation. Indeed, the small amount of 'input' that is possible, while still cushioned in-utero appears to have a depressant rather than an excitatory effect on the foetal CNS driving the infant deeper into unconsciousness (Mellor et al, 2005). In contrast, intense tactile stimulation during labour and after delivery is likely to contribute to the usually rapid onset of behavioural activity and consciousness in newborn human infants.

Thus, by the time of birth, a heel prick brings about a 'whole body movement' consisting of wriggling, rolling, and simultaneous responses of the arms and legs. Under normal circumstances, this chaotic response becomes tailored to an individual stimulus by the development of inhibitory processes. The nociceptive spinal reflex system performs highly precise sensorimotor transformations, the specificity of which is attained during early development and has been shown to be learning

dependent. In their study of the heat-nociceptive tail withdrawal reflex, Waldenstrom et al showed that under normal conditions, erroneous withdrawals (movements towards the stimulation) decreased significantly between P10 and P21. However, the improvement was completely blocked by anaesthetising the tail during the adaptation period, showing the change to be experience dependent. Interestingly, the adaptation did occur if the tail was protected only from noxious input, allowing normal tactile experience, thus nociceptive input is not a prerequisite and tactile receptors can be used to guide nociceptive synaptic organisation (Waldenstrom et al, 2003). Indeed, related work from the same group demonstrated that tactile feedback from spontaneous muscle twitches during sleep are sufficient to modify sensorimotor transformation in young rats (Petersson et al, 2003).

Research in human neonates has shown a clear relationship between postconceptional age (PCA) and cutaneous withdrawal threshold, with the mean threshold at 29 weeks being a quarter of that at 41 weeks (Andrews & Fitzgerald, 1994). As well as having an exaggerated magnitude and a low initiation threshold, neonatal nociceptive responses may be triggered by a seemingly innocuous stimulus, such as light touch both in rat pups and premature human infants (Fitzgerald et al, 1988). These responses can also be 'wound up' by repeated stimulation in the very young (under 29-35 weeks in human infants, or P8 in the rat) (Andrews & Fitzgerald, 1994). Although stronger reflexes do not necessarily mean more pain, they reflect the relative lack of normal inhibitory control exerted by higher brain structures in the mature nervous system.

1.3 Chronic Pain During Development

The immature nervous system is in a state of flux, and as such is particularly vulnerable to external insults.

1.3.1 Inflammation and Tissue Injury

Peripheral tissue injury, such as that caused by repeated heel lance blood sampling, in premature infants leads to a 50% reduction in cutaneous flexor reflex threshold (Fitzgerald, 1988). Additionally, local limb tissue damage of the contralateral limb causes an ipsilateral reduction in this threshold (Andrews & Fitzgerald, 1999). Cutaneous sensory neurons undergo considerable growth and re-organisation during postnatal growth (Reynolds et al, 1991). Tissue damage in the early postnatal period causes profound and lasting sprouting of local sensory nerve terminals, causing a persistent area of hyperinnervation (Reynolds & Fitzgerald, 1995). This hyperinnervation is greatest when the wound is performed at birth, with the magnitude of the effect decreasing with postnatal age. Neonatal cutaneous sprouting comprises both C fibres and Aδ fibres, whereas the smaller magnitude response in more mature animals comprises only small diameter C fibres. It is therefore possible that Aδ fibre sprouting accounts for the difference, and only occurs at a critical time in development. Ren and colleagues have investigated the effect of a brief neonatal inflammatory insult over a lifetime recently, by applying a carrageenan (short acting inflammation) plantar injection and following up the animals until 125 days of life (well after the animals reach maturity). They were able to reproduce the previously described hyperalgesia on re-inflammation, but noted that in the longer term (post-puberty) the animals were hypoalgesic at rest. Interestingly, the hypoalgesia was

found in both the ipsi- and contralateral paws of treated rats (implying a global process), whereas the hyperalgesia to stimulation was unilateral (suggesting a more segmentally limited effect). Both effects were still present at 120 days, with no appreciable extinction (Ren et al, 2004). Thus, bearing in mind the large number of invasive procedures undergone by premature infants during their period of intensive care, the implications of these observations are potentially far reaching, and studies of the after-effects of neonatal intensive care may need to follow the children to adulthood to appreciate the full picture.

1.3.2 Nerve Damage

The anatomy of the developing nervous system is also much more vulnerable to peripheral nerve damage than is the case in the adult. Ligation of the sciatic nerve in the infant rat leads to the death of 75% of axotomized neurons, compared to 30% in adult rats (Himes et al, 1989). As a result of cell death in the dorsal root ganglion, the central terminals of the adjacent intact neurons sprout in the spinal cord to occupy areas normally exclusive to the damaged nerves (Reynolds and Fitzgerald, 1992). These new sprouts form inappropriate connections with dorsal horn cells in areas far removed from their normal termination area, causing a permanent distortion of the nervous system with a greater proportion than normal being dedicated to inputs from the damaged area (Shortland and Fitzgerald, 1991). Despite these observations, a relative lack of neuropathic pain behaviour is seen in very young animals following chronic constriction injury or spared nerve injury, suggesting a remarkable ability in neonates to compensate for the sensory consequences of nerve injury (Howard et al, 2005). This is consistent with the good outcomes found following intra-partum brachial plexus injury compared to similar trauma in older patients (Anand & Birch,

2002). The properties of the immature nervous system described above all relate to the greater plasticity of the developing nervous system, and the mechanisms of the differences observed might themselves eventually yield clues to the aetiology of pathological pain states.

1.4 Long Term Effects of Neonatal Pain

In view of the phenomena described above, it is no surprise that the long-term impact of early neonatal pain can be enormous. Neurologically intact premature infants with birth weights of under 750g are at high risk of neurobehavioural dysfunction and poor school performance as a result of early sensory experiences in intensive care (Hack, 1994). In another study, infants with a birth weight of <1 kg were assessed at 18 months and found to have significantly lower pain sensitivity compared to controls (Grunau et al, 1994). Even in term infants, neonatal circumcision without analgesia results in increased pain responses during subsequent routine immunisation, months later (Taddio et al, 1997). In animal studies, neonatal rats exposed to 4 heel lances per day in the first week of life were found to be more sensitive to quantitative pain assessment at 16 and 22 days of life, later in adulthood these rats had an increased preference for alcohol, as well as increased anxiety, and defensive withdrawal behaviours (Anand et al, 1999). In contrast, the hyperalgesia and allodynia observed in adult models of neuropathic pain are less pronounced in young animals, a study of 24 patients following neonatal brachial plexus injury revealed no evidence of chronic pain behaviour or neuropathic syndromes (Anand & Birch, 2002). The patients also exhibited normal mechanical, vibration and thermal sensory thresholds, and the authors suggest that their relatively complete recovery may be due to later maturation of the injured fibres and the plastic nature of the CNS

in the perinatal period. Overall, the consequences of neonatal pain are not only a very real issue in the acute setting, but in view of the vulnerability of the early postnatal nervous system, may lead to a legacy of long term suffering.

1.5 The Developmental Anatomy of Pain Pathways

1.5.1 Peripheral Terminals

Sensory nerve fibres terminating in nociceptive or low threshold mechanoreceptive endings grow out from the dorsal root ganglion prenatally and innervate the skin in a proximo-distal manner. They reach the most distal skin of the foot by the second trimester in man, and by birth in the rat (Reynolds et al, 1991). The large diameter A fibres develop first, followed by the arrival of smaller diameter C fibres. C fibres fall in to 2 groups: those that contain neuropeptides (substance P and caltonin gene related peptide, CGRP) and express the neurotrophin receptor for nerve growth factor (NGF), tyrosine receptor kinase (trkA); and those that bind the isolectin IB4 and express receptors for the neurotrophin glial cell derived neurotrophic factor (GDNF). It is interesting to note that IB4+ve C fibres mature postnatally in the rat, and so the majority of C fibres at birth are trkA+ve and peptidergic (expressing CGRP) (see Chapter 2, figs 2.2 & 2.3) (Bennett, 1996). C fibre polymodal nociceptors (responding to mechanical, thermal, and chemical noxious stimuli) are fully mature in their thresholds, pattern, and frequency of firing by birth. High-threshold Aδ mechanoceptors (responding maximally to noxious mechanical rather than chemical or thermal stimulation) can be identified at birth but their peak firing frequencies are lower than in adults. Low-threshold Aβ mechanoceptors (which respond to touch or brush) are relatively the least mature at birth with low firing frequency and amplitude of response (Fitzgerald, 1987). At the same time that

sensory neurons are 'being born' and forming cutaneous plexi, they are also being 'pruned' as a result of apoptosis or programmed cell death (Coggeshall et al 1994). Survival of a particular neuron is dependent on its access to the appropriate neurotrophic factors. An example of this is nerve growth factor (NGF), produced by the skin (and other target organs), which is essential for the survival of trkA expressing peptidergic C nociceptive neurons (Ruit et al, 1992). Pathological reduction in the expression of NGF in man is implicated in a rare sensory and autonomic neuropathy known as Congenital Insensitivity to Pain (Indo et al, 1996). Conversely, an excess of NGF (or brain derived neurotrophic factor, BDNF) leads to skin hyperinervation (Albers et al 1994). Neurotrophins also have a role in the differentiation and maturation of nociceptors, in turn dictating their mechanical properties (Koltzenburg, 1999). The thresholds and receptive field sizes of cutaneous mechanosensitive primary afferents are generally the same in the adult and the neonatal rodent (Fitzgerald 1987); therefore, any perceived differences are likely to be due to changes in central processing (see below).

1.5.2 Spinal Cord

The spinal cord develops ventrodorsally, with motoneurons developing first followed by intermediate neurons, deep dorsal neurons and lastly the neurons of the substantia gelatinosa (SG, Laminae I & II) (Altman & Bayer, 1984). The growth of A and C fibres in to the cord is somatotopically precise (Fitzgerald, 1987), presumably following on from earlier foetal organisation within the dorsal root ganglia (DRG). The laminar architecture however initially exhibits an immature pattern, with Aδ afferents (restricted to laminae III & IV in adults) extending dorsally as far as laminae I & II (Fitzgerald et al 1994). This is followed by a gradual withdrawal from

the superficial laminae over the first 3 weeks of life (in the rat pup), culminating in the adult distribution. There is also evidence that the relative proportions of Aβ and Aδ fibres innervating the substantia gelatinosa (SG) region is reorganised following maturation. Patch clamp recordings suggest that Aβ fibres are the predominant input to the immature SG (up to P23), with Aδ fibres being substituted at a later stage (Park et al, 1999). Conversely, C fibres grow directly into laminae I & II, and for a time both types of fibres co-exist in the superficial dorsal horn. Indeed the arrival of C fibres into the superficial laminae appears to drive the retraction of A fibres to the deeper laminae, possibly by way of a competitive interaction (Nakasutka et al, 2000). When this competition reaches equilibrium, neural connections attain a stable state and thus anatomical plasticity is reduced (Fitzgerald et al, 1994). Indeed, neonatal administration of capsaicin (which destroys the majority of C fibres) leaves A fibres located more superficially than in normal animals (Shortland et al, 1990). Within the dorsal horn, projection neurons develop in advance of local interneurons (Bicknell & Beal, 1984), consistent with observations that some inhibitory mechanisms attributed to interneuronal activity appears to be absent at this stage of development (Fitzgerald, 1991). Projection neurons such as the spinothalamic and spinocerebellar tract neurons are generated as distinct populations at E13-E15 in the rat foetus, and those with the longest axons are generated first (Beale & Bice, 1994).

The cutaneous receptive fields of dorsal horn neurons are larger in newborn rats, undergoing progressive refinement over the first 2 weeks of life (Jennings & Fitzgerald, 1998). It remains largely unknown how the excitatory and inhibitory inputs of a single developing neuron are refined in a co-ordinated manner. Indeed recent work by Tao & Poo, using whole cells recording from Xenopus tectal neurons,

shows that although these inputs are well matched in the mature nervous system, the topography in immature neurons shows some disparity between inhibitory and excitatory receptive fields (Tao & Poo, 2005). Furthermore, they were able to demonstrate that the maturation in topography was dependent on $GABA_A$ receptor activity, without which there was significant impediment of this refinement.

The combination of large receptive fields and dominant A fibre input contribute to the exaggerated low-threshold cutaneous reflexes observed in the newborn rat and human. The postnatal maturation of synaptic connections between afferent C fibres and SG cells takes place over the first two weeks of life, with C fibre stimulation being unable to directly evoke spike activity in the spinal cord before this stage (Fitzgerald & Jennings, 1999). A diffuse, non specific response can be recorded which appears to arise from widespread release of substance P and other neurochemicals from C fibre terminals, rather than from specific, synaptically initiated excitation, and can be mediated from many neighbouring dorsal roots (Charlton & Helke, 1986). Despite this, there is evidence that the failure of C fibre electrical stimulation to evoke action potentials in superficial dorsal horn cells before P10, may not necessarily reflect a total absence of C fibre synapses during this period. Baccei et al used chemical stimuli (capsaicin and menthol) on patch clamped neurons to demonstrate the presence of nascent C fibre synapses in the rat superficial dorsal horn from birth (Baccei et al, 2003). However, the excitatory postsynaptic output from these connections was shown to increase greatly between P5 and P10; and the relative functional absence of output from these synapses in the first days of life in vivo, is likely to reflect either their small number, or the fact that they may to be too 'weak' to depolarise the postsynaptic membrane under physiological

conditions. In contrast, responses to stimuli with a strong A fibre component, such as the formalin test, produce clear reflex activity and SG c-fos activation from birth (Fitzgerald & Gibson 1984).

1.5.3 Higher Centres

Reflex responses to pain are a useful but crude guide to the experience of pain itself. This involves the input of higher centres and consequently is a much more complex process. Dorsal horn projections reach the thalamus and project onwards to the cortex from E19 in the rat and 22-34 weeks in the human foetus (Bicknell & Beal, 1984; Fitzgerald et al, 1991). Around the time of birth in rat pups, many thalamo-cortical synapses are silent, and are converted to functional glutamatergic synapses by activity dependent mechanism around P8-9. The onset of equivalent potentials in humans is around 29 weeks gestation (Klimach & Cooke, 1988). Electrophysiological analysis of cortical neurons at P7, shows them to be arranged in columns (as in the adult), but to have much bigger receptive fields (Armstrong-James, 1975). The rodent cortex remains immature for 6 weeks, and the human brain for several years. The 'complete' pain experience, involving higher inputs, may be indirectly assessed by the use of behavioural investigations. In human infants, this is achieved by the use of validated pain scales, looking at such variables as 'facial expression', 'heart rate variability', and 'patterns of cry' (Stevens et al, 1994). In 2002, Oberlander et al investigated the contribution of higher cortical centres to the experience of pain in premature infants by comparing infants at 32 weeks gestation with and without significant neurological injury (grade IV intraventricular haemorrhage or periventricular leucomalacia). They found no significant differences between the groups on any of the behavioural measures employed, suggesting that

higher centres may have less influence on measurable pain behaviour in significantly premature infants. True measurement of the higher cortical component of infant pain is confounded by the fact that many of the behavioural variables used may comprise a degree of reflex activity, and as such may not reflect true conscious pain responses. Future quantitative studies using near infrared spectroscopy and electro-encephalography are likely to provide an invaluable contribution to our better understanding of higher processing.

1.5.4 Segmental and Descending Inhibition

Gradual suppression or inhibition of neuronal connections is an essential feature of the developing nervous system, which takes place relatively late in the maturational process. Although, networks of descending pathways projecting from cerebral structures to the dorsal horn play a crucial role in this process, local segmental inhibition within the cord is also important. It has been long established that in the mature nervous system, focal electrical stimulation of the periaqueductal grey matter (PAG) produces a state of analgesia. This effect can be reproduced by the microinjection of opioids into the same brain region, hinting at its functional importance (Gebhart, 2004). Further study of this phenomenon revealed that these descending influences on spinal nociceptive processing rely on relays in the rostroventral medulla (RVM), which is now considered the final common output for descending influences from sites rostral in the brain (Suzuki et al, 2004). Descending influences on spinal processing are tonically active in the mature animal, and are principally inhibitory, in that nociceptive reflexes are exaggerated after transection of the spinal cord. However, electrical stimulation of the RVM has been shown to produce inhibition (40-45% of sites studied); facilitation (10-15% of sites studied); or

occasionally a biphasic output related to the intensity of stimulation (Gebhart, 2004). The different physiologically defined cells involved are known as 'ON-cells' and 'OFF-cells' and contribute respectively to descending facilitation and inhibition. Interestingly, it has been shown that peripheral tissue inflammation leads to long lasting changes in the excitability of these cells (Gebhart, 2004).

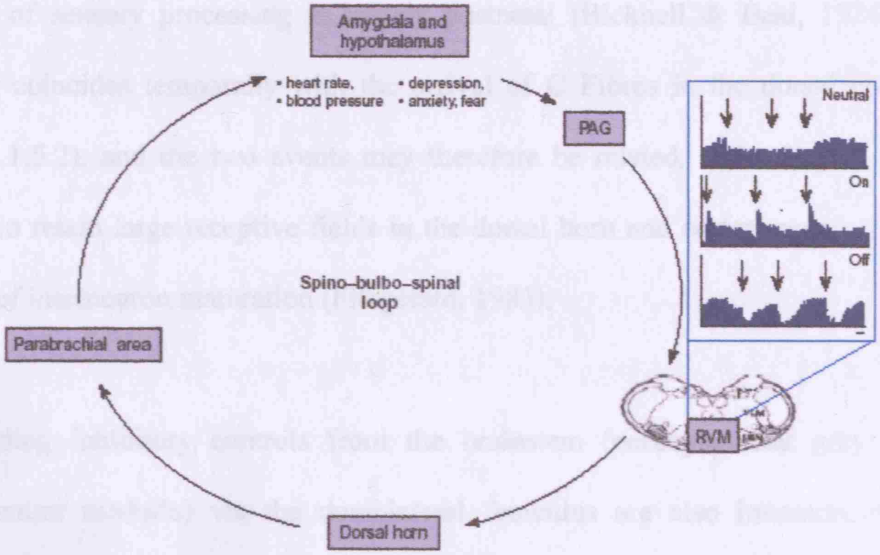


Illustration of the modulatory interaction of the dorsal horn with higher centres and the role of the RVM in descending output coordination (adjusted from Suzuki et al, 2004)

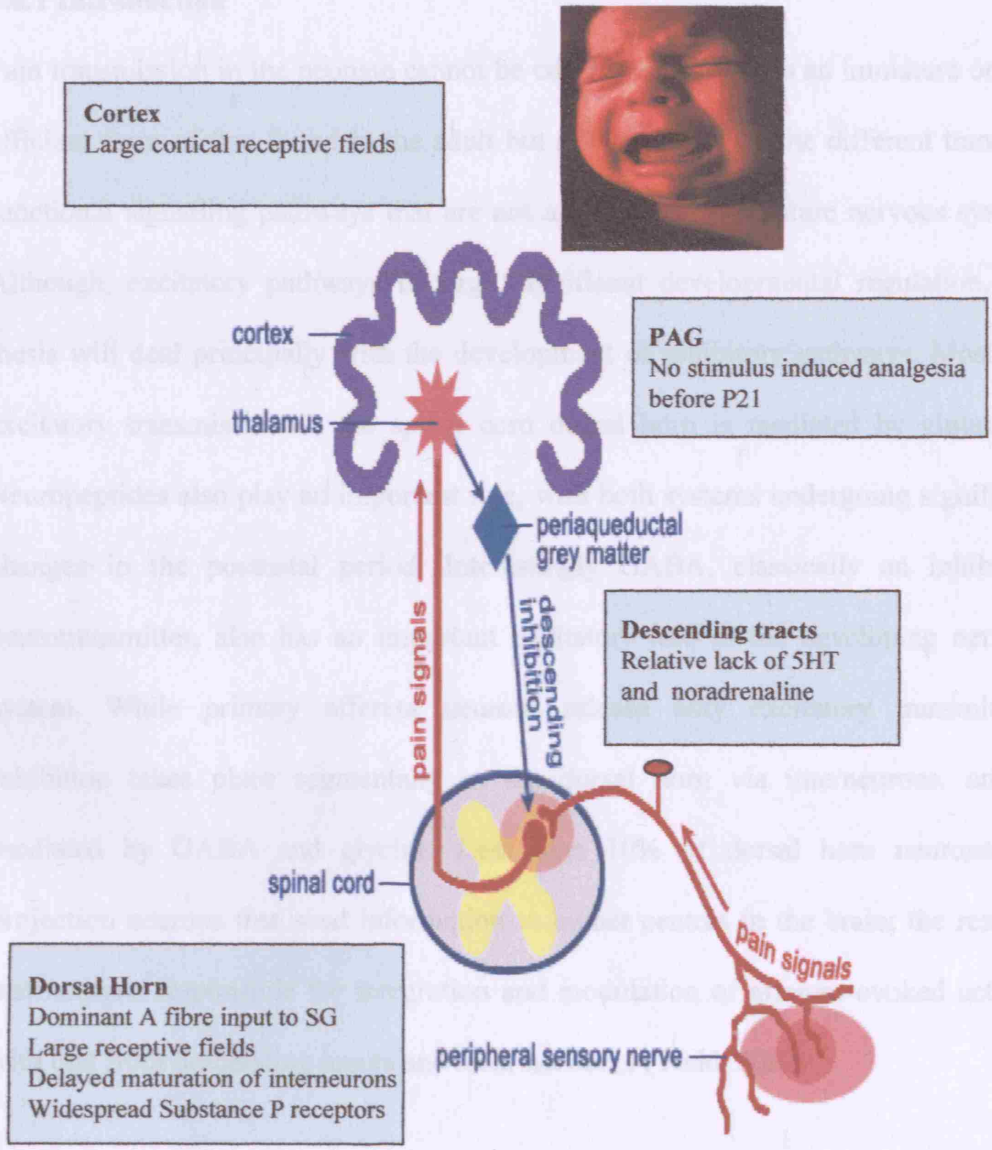
Descending inhibition is also important in the naturally activated control system known as 'diffuse noxious inhibitory control' (DNIC), a phenomenon whereby one noxious stimulus inhibits the pain produced by another (Boucher et al, 1998). Many of these inhibitory processes are postnatally regulated, as discussed below.

Some segmental inhibition is present in the rat spinal cord by the time of birth. Renshaw cell inhibition, which prevents positive feedback from occurring, when sensory neurons stimulate motoneurons, for instance, is well developed at this stage

(Fitzgerald, 1999). There is also evidence of some contralateral segmental inhibition in human infants in the first days of life. Research in premature neonates has revealed that the application of a maintained stimulus to the contralateral limb significantly inhibits withdrawal reflex responses to ipsilateral von Frey hair stimulation in infants as young as 27.5 weeks PCA (Andrews & Fitzgerald, 1994). Nevertheless, the maturation of most of the interneurons in the SG responsible for control of sensory processing is largely postnatal (Bicknell & Beal, 1984). This process coincides temporally with the arrival of C Fibres in the dorsal horn (see section 1.5.2), and the two events may therefore be related. Animals treated with capsaicin retain large receptive fields in the dorsal horn and cortex, possibly due to failure of interneuron maturation (Fitzgerald, 1983).

Descending inhibitory controls from the brainstem (periaquaductal grey matter, rostroventral medulla) via the dorsolateral funiculus are also immature at birth (Fitzgerald, 1991). The rostro-caudal development of the corticospinal tract in the human is not complete until postnatal life, as demonstrated by the presence of an extensor plantar response ('Babinski response') for the first months of life, analogous to that seen in adults following spinal injury (Andrews & Fitzgerald, 2000). Although the axons descend from the brainstem in early foetal life, they do not extend collateral branches in to the dorsal horn for some time, and are not functionally effective until P10 in the rat pup (Fitzgerald & Koltzenburg, 1986). This may be partly due to a relative lack of the neurotransmitters involved in inhibition from higher centres (5HT and noradrenaline), but is also likely to be a further reflection of the immaturity of critical interneurons in the dorsal horn itself. It has been suggested that the maturation of descending inhibition may also be C fibre

dependent; as capsaicin treated, rats have reduced inhibitory control (Cervero & Plenderleith, 1985). Cutaneous withdrawal reflex responses in human infants under 35 weeks PCA exhibit sensitisation (or 'wind up') to repeated stimulation due to the absence of the normal habituation seen in mature systems, thus reflecting a lack of inhibitory control (Andrews & Fitzgerald, 1994). Lack of effective descending inhibition can also be demonstrated by the lack of stimulus-produced analgesia from the periaqueductal grey matter before P21 in young rats (van Praag & Frenk, 1991), and more recently, Hahm and colleagues have shown a postnatal maturation of GABAergic spontaneous miniature postsynaptic currents in the RVM, implying postnatal maturation of GABAergic tone (Hahm et al, 2005). DNIC has been shown to be ineffective before P12, with reduced nociceptive output in response to a heterotropic stimulus being detectable only from P21 (Boucher et al, 1998). Thus, in conclusion, the relative lack of segmental and descending inhibition in the immature nervous system results in the absence of normal 'damping down' following a painful stimulus.



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For References see text

1.6 The Development of GABA & glycine signalling

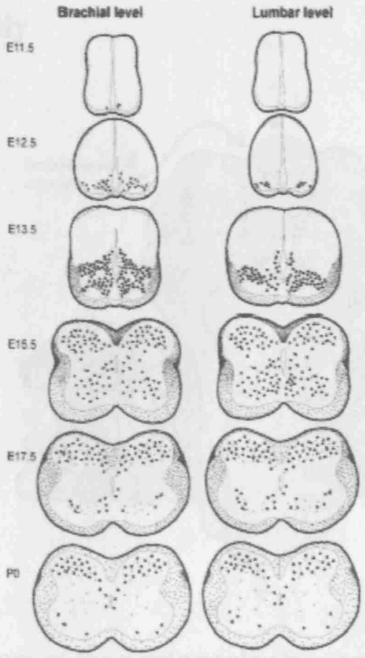
1.6.1 Introduction

Pain transmission in the neonate cannot be considered simply as an immature or less efficient form of that found in the adult but rather involves quite different transient functional signalling pathways that are not a feature of the mature nervous system. Although, excitatory pathways undergo significant developmental regulation, this thesis will deal principally with the development of inhibitory pathways. Most fast excitatory transmission in the spinal cord dorsal horn is mediated by glutamate. Neuropeptides also play an important role, with both systems undergoing significant changes in the postnatal period. Interestingly GABA, classically an inhibitory neurotransmitter, also has an important excitatory role in the developing nervous system. While primary afferent neurons release only excitatory transmitters, inhibition takes place segmentally in the dorsal horn via interneurons, and is mediated by GABA and glycine. Less than 10% of dorsal horn neurons are projection neurons that send information to higher centres in the brain; the rest are interneurons responsible for integration and modulation of afferent evoked activity with that from descending inputs and local networks (Todd, 2002).

1.6.2. GABA

GABA (Gamma-aminobutyric acid) is the most ubiquitously expressed inhibitory transmitter in the mammalian CNS. Its principle role is via short interneurons, with the only long GABAergic tracts being those running to the cerebellum and striatum. In the context of pain modulation, important groups of GABAergic interneurons exist in the periaquaductal grey matter (PAG), the rostroventral medulla (RVM), and

the spinal dorsal horn, as well as in higher cortical centres. GABA plays a pivotal role in many aspects of neuronal development, with its receptors being present at synapses before any others. GABA is transiently over expressed in the developing cord, with 50% of neurons being GABA positive in the first 2 weeks, compared with 20% by week three (Schaffner et al, 1993). The rate limiting synthetic enzyme for GABA, glutamic acid decarboxylase (GAD), is widely distributed in the spinal cord by birth and its levels can be shown to increase post-natally until P14 when the levels fall with the enzyme becoming concentrated in the superficial dorsal horn (Ma et al, 1994).



GABA labelling of cell bodies in mouse spinal cord during development, from Allain et al, 2004

GABA immunoreactivity in cell bodies is first detectable at E11.5, in the mouse spinal cord, at the branchial level, in the marginal zone. It develops rostro-caudally, and ventro-dorsally first appearing in the dorsal horn region around E15.5. By the time of birth, GABA expression is localised to the dorsal horn, and had declined in intensity. It is also interesting to note that the early ventral expression has been

shown not to co-localise with markers of motoneurons (Allain et al, 2004). GABA immunoreactive nerve fibres also appear at around E11.5, in the lateral white matter of the brachial marginal zone. They invade the ventral marginal zone reaching the dorsal side by E15.5, and by P0 the number of positive fibres in the white matter decline (Allain et al, 2004).

GABA acts at postsynaptic $GABA_A$ and $GABA_C$ receptors (pentameric structures linked to chloride channels, see below), and pre- and postsynaptically at $GABA_B$ receptors, which are metabotropic receptors suppressing calcium influx and reducing transmitter release pre-synaptically as well as activating potassium channels postsynaptically.

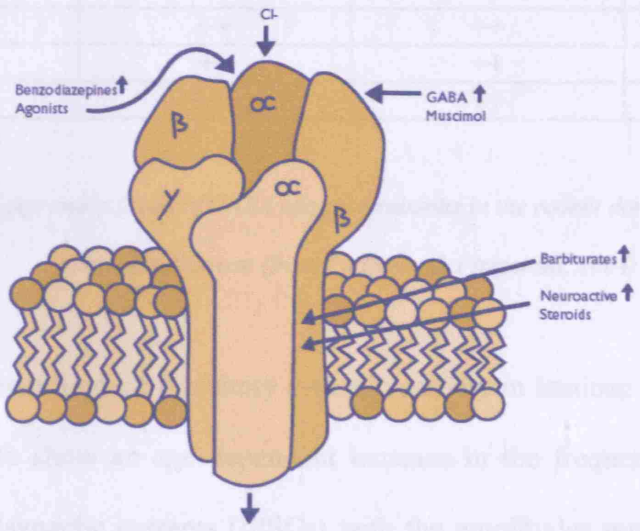


Diagram of the pentameric structure of the $GABA_A$ receptor

Postsynaptic $GABA_B$ mediated inhibition is poorly developed in the immediate postnatal period. However, presynaptic $GABA_B$ activity is well developed at birth and plays a crucial role in modulating post-synaptic activity by depressing transmitter release at early postnatal stages (Li et al, 2004). The $GABA_A$ receptor is

made up of combinations of α, β, γ, δ, ε, π, and θ subunits. In the neonatal rat dorsal horn, the most prominent subunits are α_2, α_3, β_3, and γ_2. $GABA_A$ receptors undergo changes in subunit composition during development, allowing them to react in different ways to presynaptic stimuli (see table below), the γ_2 subunit is the most highly expressed in the immature cord, with levels falling to adult values after the two first weeks of life (Mehta & Ticku, 1999). This particular subunit has been shown to be critically important in the post-synaptic clustering of GABA receptors, by its interaction with the scaffolding protein Gephyrin, (Essrich et al, 1998). Gephyrin will be discussed in more detail in section 1.7.

GABAa	P0	P14	Adult
α_2	++	++	++
α_3	++	++	++
β_3	++	++	++
γ_2	+++	++	++

Comparison of expression levels of GABA receptor subunits in the rodent dorsal horn during post-natal development (from Pattinson & Fitzgerald, 2004)

Patch clamp recordings of inhibitory synaptic activity in laminae I & II over the first 2 weeks of life show an age dependent increase in the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) with the amplitudes remaining unchanged. GABAergic mechanisms appear to underlie the majority of spontaneous and evoked transmission at putative inhibitory synapses during this period, with little contribution from glycine (Baccei and Fitzgerald, 2004). The decay time of these GABAergic mIPSCs is longer than seen in adults. This has been shown to be due to the tonic production of 5α-reduced neurosteroids, which confer slow kinetics on the mIPSCs in lamina II of the neonatal spinal cord (Keller et al, 2004).

Changes in the intracellular cation concentration of $GABA_A$ receptors during development also confer important functional changes during maturation (Rivera et al, 1999). In many areas of the developing brain, $GABA_A$ receptors behave in an excitatory manner, before switching to their usual inhibitory function at a later stage of postnatal development. This was first described in chick embryonic spinal neurons in culture, but has subsequently been shown to be a widespread phenomenon (Ben Ari, 2002). The depolarising actions of early $GABA_A$ receptor activation are due to the relatively high intracellular chloride concentration of immature neurons with resting potentials that are significantly more negative than the equilibrium potential (or reversal potential) for chloride. As development proceeds, the neuronal chloride concentration decreases, allowing the effect of GABA to become progressively inhibitory (Owens and Kriegstein, 2002). The required active extrusion of chloride is mediated by the neuron-specific potassium-chloride cotransporter KCC2, which has been shown to be developmentally regulated (Rivera et al, 1999). KCC2 will be discussed in more detail in section 1.8.

During early postnatal development (<P23), certain inhibitory synapses are able to co-detect GABA and glycine; however, in later life the synapses can detect either GABA or glycine but not both (Keller et al, 2001). It has been suggested that co-transmission could activate pre-synaptic $GABA_B$ receptors and thus provide negative feedback control on transmitter release (Lim et al, 2000). The developmental fine-tuning of this particular property of immature neurons appears to be region specific. All neonatal lamina I & II IPSCs are either GABAergic, glycinergic or mixed, whereas after P23 lamina I tends to have only glycinergic IPSC, but lamina II IPSCs

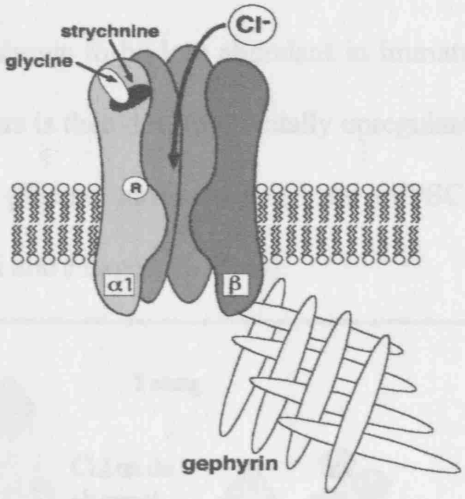
are split approximately equally between GABAergic and glycinergic currents. The sequence of events leading to the formation of single or mixed inhibitory synapses is not fully understood. In spinal cord cultures, it has been found that $GABA_A$ receptor clusters are formed before clusters of gephyrin and glycine receptors, suggesting that the former may be responsible for synapse maturation (Dumoulin et al, 2000). $GABA_A$ receptors have been shown to cluster opposite glutamatergic and glycinergic only synapses, glycine receptors on the other hand do not form post-synaptic densities when spinal neurons are only innervated by GABAergic neurons (Levi et al, 1999). In summary, GABA appears to behave as a developmental signal as well as a classic neurotransmitter, during the early stages of the maturation of the nervous system.

1.6.3 Glycine

Glycine is found in particularly high concentrations in the grey matter of the spinal cord as well as in the brainstem. Indeed glycine receptors are the most abundant inhibitory receptors in the spinal cord, where they regulate neuronal excitability. Glycinergic transmission is relatively sparse in higher centres, with many cortical glycine receptors being extra-synaptic and of uncertain function (Aguayo et al, 2004). Glycine transmission is also predominantly mediated via inhibitory interneurons. Like $GABA_A$ receptors, glycine receptors are composed of a number of subunits arranged in a pentomeric fashion and show clear developmental expression profiles. In the neonatal cord, the α2 subunit is expressed throughout the grey matter, forming heteromers with the β subunit (Watanabe, and Akagi, 1995). Although the expression of the β subunits remains high, α_2 subunits are strongly down regulated during the first 3 weeks of life, to be replaced with α1 subunits, (see table below).

Glycine	P0	P14	Adult
α_1	+	+++	+++
α_2	+++	+	-
β	++	+++	+++

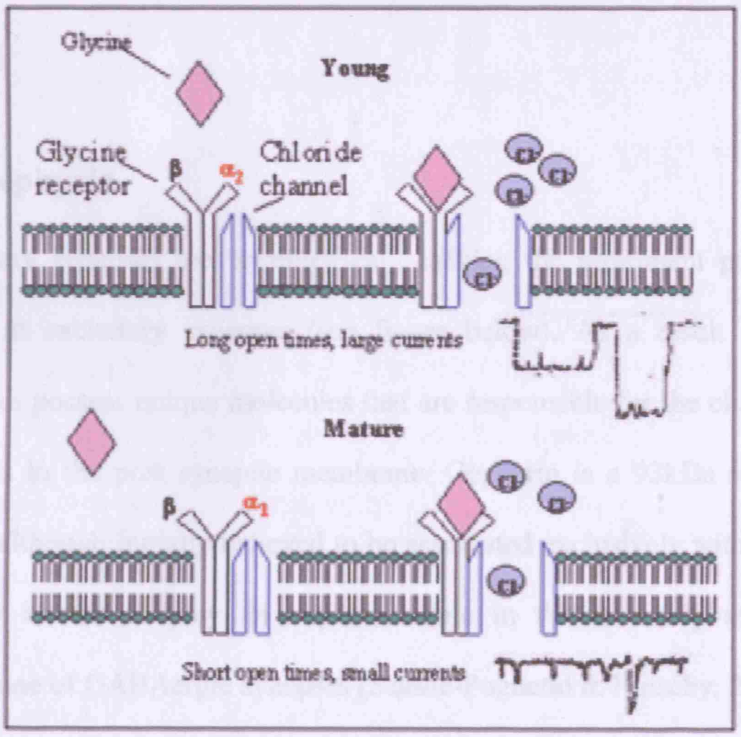
Comparison of expression levels of glycine receptor subunits in the rodent dorsal horn during post-natal development (from Pattinson & Fitzgerald, 2004)



Structural diagram of a glycine receptor

As with other categories of receptors, individual glycine receptor subunits show very different kinetic properties. The mean open time for α_2 subunits, expressed as functional homomeric glycine-gated chloride channels in *Xenopus oocytes,* is 174 milliseconds whereas that of α1 subunits is only 2.38 milliseconds. Accordingly, there is a developmental reduction in mean open time for glycine channels in spinal neurons and a shortening of the decay time constant of IPSCs in dorsal horn cells in vitro (Aguayo et al, 2004). Glycine receptor α subunits are also inhibited by strychnine with distinct affinities. Consequently, mature neurons have a higher strychnine affinity, as a result of their expression of α_1 rather than α_2 subunits. Picrotoxin (non-competitive antagonist at glycine receptors) conversely, has a higher affinity for α_2 receptors, causing greater inhibition at immature glycine receptors,

which reduces with development (Aguayo et al, 2004). Glycine receptors in cultured spinal neurons are not evenly distributed in the cell membrane. Immunohistochemical analysis has revealed that receptors form clusters in the post-synaptic membrane, and a positive correlation between IPSC amplitude and cluster size has been reported (Lim et al, 1999). These gephyrin dependent clusters occur extra-synaptically as well as synaptically (co-localized with Synapsin) with synaptic clusters having been shown to be less abundant in immature neurons. The size and number of these clusters is then developmentally upregulated, corresponding with the reported alteration in glycine current density and mIPSC amplitude in developing spinal neurons (Baccei and Fitzgerald, 2004).



Demonstration of the maturational changes in glycine receptor subunit composition and their effect on postsynaptic currents

Immature glycine receptors can induce rapid depolarisation in a manner analogous to that of neonatal $GABA_A$ receptors. This is also as a result of high neonatal intracellular chloride concentrations due to a relative lack of KCC2 (discussed in

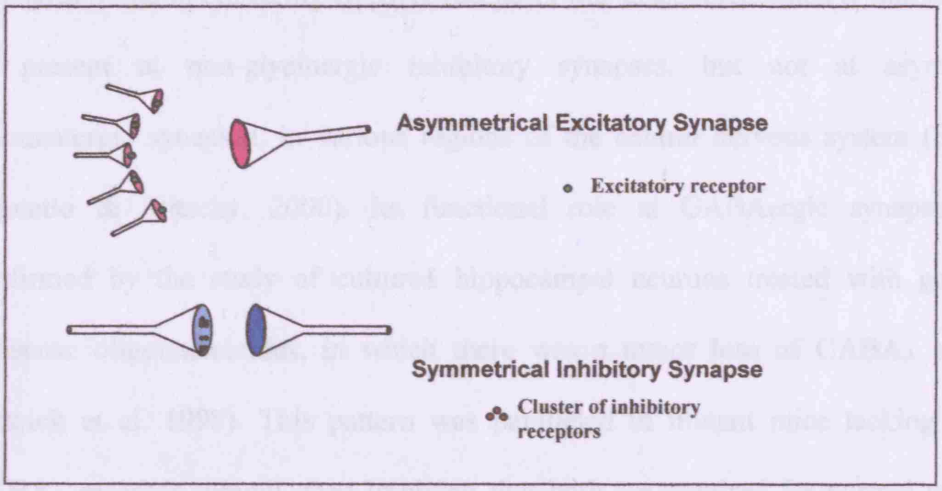
detail in section 1.8). Interestingly, blockade of immature glycine receptors with strychnine can be shown to cause an increase, rather than a decrease in neuronal excitability (Tapia et al, 2001). Finally, it has been suggested that glycinergic activity may have an important role in the modulation of neuronal outgrowth in the immature nervous system. This has recently been investigated in cultured neurons where it was found that reducing glycinergic activity with strychnine produced a significant increase in neurite outgrowth, dendritic spines, and overall activity mediated by glycine, GABA and AMPA receptors. However, since this trophic effect was blocked by the $GABA_A$ antagonist bicuculline, a modulatory interplay between GABA and glycine transmission appears the most likely overall mechanism (Tapia, 2001).

1.7 Gephyrin

Inhibitory synapses are 'symmetrical', lacking the prominent postsynaptic density found at excitatory synapses (see figure below). As a result of this, inhibitory synapses possess unique molecules that are responsible for the clustering of receptor proteins in the post synaptic membrane. Gephyrin is a 93kDa scaffolding protein, which although initially believed to be associated exclusively with glycine receptors, is now known to play an important role in the assembly at the postsynaptic membrane of GABAergic synapses (Sassoe-Pognetto & Fritschy, 2000).

1.7.1 Introduction

Gephyrin was first found to co-purify with the glycine receptor and to be located on the postsynaptic side of glycinergic synapses (Triller et al, 1985).



Evidence for its functional role came from experiments involving the depletion of gephyrin by antisense oligonucleotides in cultured spinal neurons (Kirsch et al, 1993); or by gene targeting in mice (Feng et al, 1998) both of which prevented the formation of glycine receptor clusters. Gephyrin (-,-) mice die within a day of birth, suggesting that it is not essential for embryonic development, but is crucial for postnatal survival. The gephyrin (-,-) neonates appear normal at birth, but fail to suckle or vocalise, and assume a rigid hyper-extended posture in response to mild tactile stimuli. These mice become increasingly sensitive to tactile stimuli, before developing apnea by 12 hours after birth, as a result of the effect of gephyrin deficiency on motoneurons (Feng et al, 1998).

1.7.2 Function

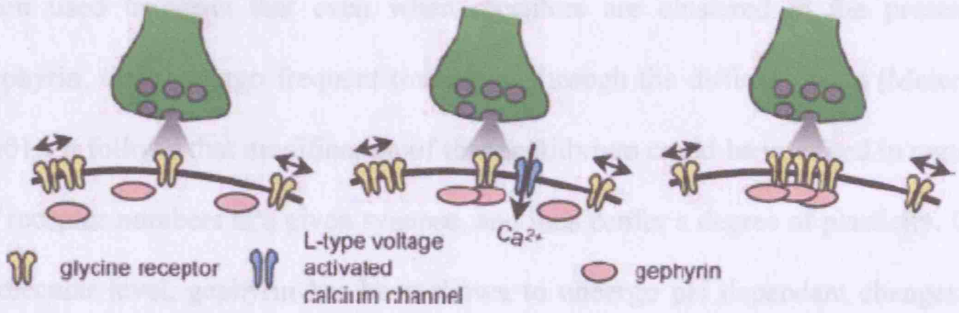
Gephyrin is effective in conferring stability by virtue of its binding with elements of the cytoskeleton, including tubulin. Indeed disruption of microtubules or microfilaments has been shown to impair glycine receptor clustering (Kirsch & Betz, 1995). Gephyrin interacts with a large cytoplasmic loop of the β subunit of the glycine receptor, making this subunit essential for the clustering of receptors.

Gephyrin's role in clustering $GABA_A$ receptors was discovered when it was found to be present at non-glycinergic inhibitory synapses, but not at asymmetric glutamatergic synapses, in various regions of the central nervous system (Sassoe-Pognetto & Fritschy, 2000). Its functional role at GABAergic synapses was confirmed by the study of cultured hippocampal neurons treated with gephyrin antisense oligonucleotides, in which there was a major loss of $GABA_A$ clusters (Essrich et al, 1998). This pattern was paralleled in mutant mice lacking the γ_2 $GABA_A$ receptor subunit, thus implying that both are required for normal receptor clustering. Despite its effect on receptor density, the absence of gephyrin does not prevent assembly and cell surface targeting of $GABA_A$ receptors, its precise role is likely to be stabilisation of receptors in the postsynaptic membrane and prevention of lateral diffusion / internalisation. Further studies in cultured hippocampal neurons have revealed that although gephyrin (-,-) mice can not form any functioning glycine receptor clusters, $GABA_A$ receptor postsynaptic collections could still occur (in reduced numbers) and GABA induced miniature IPSCs could still be recorded, all be it at a reduced amplitude (Levi et al, 2004). Other proteins, such as GABA receptor associated protein (GABARAP), are also involved in the cellular localisation of $GABA_A$ receptors. This molecule has been shown to interact biochemically with γ_2 $GABA_A$ receptor subunits, and with gephyrin, however, it is concentrated in the intra-cellular compartment rather than in postsynaptic clusters, and may therefore have a role in receptor sorting / targeting mechanisms (Kneussel et al, 2000). In the cerebellum and hippocampus, $GABA_A$ receptor clusters co-localise with dystrophin, the protein that is responsible for muscular dystrophy, as well as with gephyrin. In a mouse model of muscular dystrophy (dystrophin-null mouse), $GABA_A$ receptor clusters are reduced only in areas that are dystrophin rich in the intact animal, and

gephyrin clusters remain unaffected (Brunig et al, 1999). The apparently subtly different roles played by gephyrin at GABA and glycinergic synapses, are reflected in the functional impact of gephyrin antisense oligoneucleotides on glycinergic and GABAergic synaptic transmission in spinal neurons. Glycinergic mIPSCs are reduced in both frequency and amplitude, whereas GABAergic neurons are only reduced in frequency, but have an altered response to benzodiazepines and zinc (van Zundert et al, 2005).

1.7.3 Constructing Inhibitory synapses

The mechanisms behind the construction of inhibitory synapses are not yet fully elucidated. Gephyrin forms aggregates first at non-synaptic loci (Dumoulin et al, 2000), but as soon as vesicular inhibitory amino acid transporter (VIAAT) decorates presynaptic inhibitory boutons, gephyrin predominantly accumulates opposite presynaptic terminals. Coinciding with the expression of a particular pre-synaptic neurotransmitter phenotype, glycine and/or GABA receptors then associate in the post-synaptic neuron, with these preformed post-synaptic anchor structures (Meier et al, 2000).



Putative mechanism of inhibitory synapse assembly. In the face of high intracellular Cl^-, glycine receptor activation is depolarising. This depolarisation, along with calcium influx via voltage gated channels triggers gephyrin to accumulate and trap the freely diffusing receptors. Adapted from Meier et al, 2000.

Thus, during the development of the spinal cord, gephyrin clusters form before glycine receptor clusters. It has therefore been hypothesised that un-clustered immature glycine receptor activation, causing depolarisation triggers calcium influx, driving the formation of gephyrin clusters, which in turn 'trap' and concentrate glycine receptors opposite active pre-synaptic terminals (see diagram above). However, blockade of glycinergic activity with strychnine prevents glycine receptor clustering, but not gephyrin clustering. Denervation in the mature animal, on the other hand, does disrupt gephyrin clustering suggesting that other pre-synaptic derived factor(s) must be required (Seitanidou et al, 1992). Whereas glycine receptor clustering requires receptor activation, this does not appear to be the case for $GABA_A$ receptors (Craig et al, 1994). Gephyrin and $GABA_A$ receptors are also able to co-cluster after nerve injury in the mature animal, discounting early GABAergic excitation as an essential component of synapse formation (Kirsch & Betz, 1998).

More recently, it has become clear that clustered receptors in the postsynaptic membrane are in an active state of equilibrium, with other freely diffusing receptors. Gephyrin induces long-term periods of confinement to membrane clusters, and in its absence most receptors are freely diffusing. Single particle tracking techniques have been used to show that even when receptors are clustered in the presence of gephyrin, they undergo frequent transitions through the diffusive state (Meier et al, 2001). It follows that modification of this equilibrium could be involved in regulation of receptor numbers at a given synapse, and thus confer a degree of plasticity. On the molecular level, gephyrin has been shown to undergo pH dependant changes in its three dimensional structure, which allow reversible post synaptic scaffolding for glycine receptor recruitment (Sola et al, 2004). Further studies of the intracellular

milieu; have suggested that in certain circumstances, glycine receptors are associated with gephyrin on their way to the cell surface and that this association increases the efficiency of the accumulation of glycine receptors at the plasma membrane (Hanus et al, 2004).

1.7.4 Activity Dependence

Activity dependence appears to have a role in this synaptic plasticity, Gonzalez-Forero and colleagues injected high dose tetanus toxin in to the lateral rectus muscle of a cat, at a dose sufficient to reduce neuronal firing. They found a reduction of 60% in the density of gephyrin clusters in the post-synaptic membrane, with a corresponding presynaptic reduction in vesicular GABA transporter (VGAT) (Gonzalez-Ferero et al, 2004). Similarly, it has been shown that facial nerve axotomy leads to a sharp decrease in glycine receptor clusters and gephyrin in the facial nucleus, which is maximum at 8 days and recovered by day 60 post injury (Eleore et al, 2005). In this case, however, the authors did not find a matching reduction in VIAAT immunoreactivity pre-synaptically.

1.7.5 Non-structural Roles

Aside from its structural role, gephyrin has been found to interact with other protein including RAFT1 (an immunophilin that acts as a regulator of mRNA translocation), and may also therefore fulfil a further modulator role for changes in synaptic activity and structure (Sassoe-Pognetto & Fritschy, 2000). Gephyrin is also involved in the biosynthesis of the molybdenum co-factor which is essential for all molybdenum dependent enzymes in mammals (Feng et al, 1998).

1.7.6 Expression in Human CNS

Thus, among other roles, gephyrin is a major component of the post-synaptic apparatus of symmetrical synapses in brain and spinal cord, involved in the stabilisation of glycine and $GABA_A$ receptor clusters in the post-synaptic membrane. Although the data described so far relates to gephyrin's role in the rodent CNS, the distribution of gephyrin has also been studied immunohistochemically in the post-mortem human central nervous system and found to be largely similar (Waldvogel et al, 2003). Baer and co-workers have also confirmed the co-localisation of gephyrin and glycine receptors throughout the human brainstem and spinal cord in a further study (Baer et al, 2003). The phenotype of mutant mice lacking gephyrin (decribed above) mimics the condition of 'Human Hereditary Hyperekplexia' (or startle disease), a disorder classically caused by mutations in glycine receptor genes, in which some patients have recently been found to have gephyrin mutations (Rees et al, 2001). In addition, autoimmunity to gephyrin has been documented in patients with 'Stiff Man Syndrome' a disease with some similarity to Human Hereditary Hyperekplexia (Buttler, 2000), and gephyrin deficiency is being investigated in the aetiology of certain familial epilepsy syndromes (RJ Harvey, personal communication). Lastly, Gephyrin has been studied in the globus pallidus of patients suffering from Huntington's disease. The results revealed increased $GABA_A$ receptor subunit immunoreactivity and unchanged levels of gephyrin; implying that gephyrin can maintain a stable lattice even in the face of disease related changes in receptor numbers (Thompson-Vest et al, 2003).

1.7.7 Conclusion

In view of its importance to the effective functioning of inhibitory synapses in the spinal cord, gephyrin is likely to have an impact on the integrity of inhibitory systems in pain pathways. The fact that gephyrin (- , -) mice are born apparently healthy and then die within a day of birth highlights the importance of its role in early postnatal development. Gephyrin has been shown to be postnatally regulated in various regions of the central nervous system (Fallah et al, 1999), and in this thesis, its postnatal developmental expression will be studied in the dorsal horn of the spinal cord.

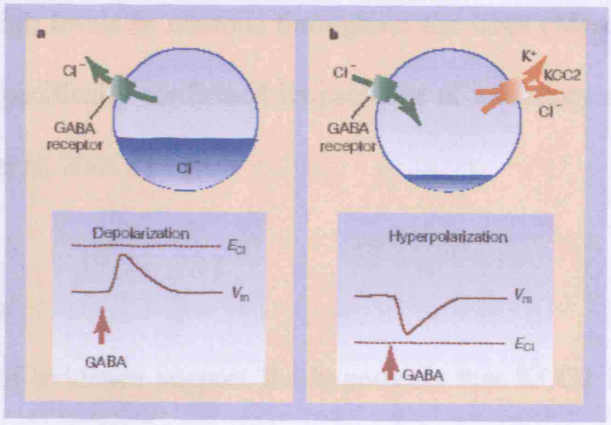
1.8 KCC2

$GABA_A$ receptor signalling is best known for its hyperpolarizing action and its role in synaptic inhibition, however, a depolarising action was recognised in some of the very earliest studies of spinal cord and developing neural tissue.

1.8.1 Introduction

In adult animals, synaptic inhibitory potentials, via $GABA_A$ and glycine receptors, are generally hyperpolarizing because these receptors are coupled to a chloride channel, and the chloride equilibrium potential is more negative than the cell's resting potential. The chloride gradient has been shown to be opposite, at an early stage in development, due to a relatively high intracellular chloride concentration, causing responses to depolarising (Ben Ari et al, 1989). This excitatory role for GABA was first proposed as early as 1963, although at that stage its mechanism remained a mystery. In the some of the first pharmacological studies on spinal primary afferent depolarisation, GABA was shown to have a depolarising and

therefore excitatory action on primary afferents (Eccles et al, 1963). Later, it was shown, in embryonic chick neuron cultures, that GABA had an excitatory role, which over time switches to the well established inhibitory action of the adult (Obata et al, 1978). This 'switch' has since been demonstrated postnatally, in acute *in vitro* mammalian preparations in a wide range of CNS regions including the hippocampus, brainstem, and cortex (Ben-Ari et al, 1989; Kullmann and Kandler, 2001; LoTurco et al, 1995). In neonatal superficial dorsal horn neurons, patch clamp recordings have shown that GABA produced depolarisation in 40% of neurons at P0-P2; with only hyperpolarisation recorded from P6-P7 onwards (Baccei & Fitzgerald, 2004).



Relationship between KCC2, intracellular chloride concentration and GABAA receptor effect: a) immature neuron; b) mature neuron, from Miles, 1999.

Over time it became clear that the reason for this qualitatively altered response to GABA was related to intracellular anion concentrations. As $GABA_A$ and glycine receptors operate by opening chloride channels in the cell membrane, the chloride gradient across the cell membrane dictates the direction of the chloride flow, and thus the polarity of the response. Immature neurons have higher intracellular chloride concentrations, which reduce during maturation to reach adult levels, accounting for

the 'switch' seen in $GABA_A$ receptor function. Over the past few years a family of cation-chloride co-transporters (SLC12 family; KCC1, KCC2, KCC3, KCC4, NKCC1) has been identified, which tightly control intracellular chloride concentration (Mercado et al, 2004). Many of these transporters molecules have important roles outside the CNS (heart, kidney), however KCC2 is neuron-specific; and NKCC1 serves an important role in CNS development despite being ubiquitously expressed (Payne et al, 1996).

KCC2 (or SLC12a5), a 140 kDA protein, was first cloned by Payne et al in 1996. In-situ hybridisation and immunohistochemical studies have shown that KCC2 is expressed at high levels in neurons throughout the CNS (Mercado et al, 2004), and Hubner et al specifically confirmed its presence at inhibitory synapses in the spinal cord (Hubner et al, 2001).

1.8.2 Function

Several lines of evidence support the hypothesis that KCC2 is responsible for the perinatal differences in GABA and glycine responses. In many brain areas, KCC2 levels have been shown to be low at birth, with a marked increase in the first week of development (Clayton et al, 1998; Lu et al, 1999). KCC2 is detectable as early as E10.5 (Stein et al, 2004), and by P15 expression is indistinguishable from adult levels throughout the nervous system (Wang et al, 2002; Stein et al, 2004). Expression in the spinal cord can be detected in the ventral motoneurons from E12, with detectable levels throughout the cord by E18 (Hubner et al, 2001). In a recent study, Stein et al suggested that KCC2 levels in the spinal cord do not change significantly beyond P3; much earlier than elsewhere in the CNS. However, in this

study the dorsal and ventral portions of the cord (Which have different embryonic KCC2 expression, and contrasting functional roles) were processed together, thus post-natal developmental differences in dorso-ventral expression would have been missed (Stein et al, 2004). Reduction in KCC2 expression in pyramidal cells from rat hippocampus using antisense oligonucleotides resulted in a marked positive shift in the reversal potential of GABA responses leading Rivera and colleagues to conclude that KCC2 was the main chloride extruder to promote fast hyperpolarizing inhibition in the brain (Rivera et al, 1999). Furthermore, in the context of pain pathways, rats treated intrathecally with KCC2 antisense oligonucleotides were found to be hypersensitive to tactile and noxious thermal stimuli (Coull et al, 2003), in a similar way to the intrathecal administration of $GABA_A$ or glycine inhibitors (Sivilotti & Woolf 1994; Ishkawa et al, 2000). Complete knockout of KCC2 in SLC12a-null mice results in early neonatal death due to apnoeic respiratory failure (Hubner et al, 2001). Zhu and colleagues examined the role of KCC2 in controlling and regulating intracellular chloride by comparing the reversal potentials of $GABA_A$ receptor-mediated chloride currents in cortical neurons cultured from wild-type and KCC2 (-,-) mice. They showed that the normal developmental decrease in intracellular chloride ion concentration in neurons is absent in mice lacking KCC2, and that chloride homeostasis in the mature neuron is also severely impaired (Zhu et al, 2005). Consistent with its role in the developmental 'switch' Hubner and co-workers used patch clamp techniques to show that GABA and glycine were inhibitory in cultured mature wild-type motor-neurons, but excitatory in neurons from KCC2-null mice. More recently, Tornberg and co-workers have developed a hypomorphic KCC2-deficient mouse retaining 15-20% KCC2 levels that is both viable and fertile, making behavioural studies a realistic proposition. The phenotype

of these mice reflected their relative lack of inhibitory tone, with increased anxiety-like behaviour and reduced seizure threshold, in the presence of normal spontaneous locomotor activity (Tornberg et al, 2005). However, in apparent contrast to these findings, the animals showed reduced sensitivity to tactile and noxious thermal stimuli. The authors postulate that this may represent the influence of descending control from supraspinal sites (rostroventral medulla and periaqueductal grey matter), where reduced hyperpolarizing GABAergic inhibition in the central pain modulatory neurons could lead to overall antinociception. Additional evidence for the causal role of KCC2 was provided by Lee and co-workers who found that transfecting immature rat neocortical neurons with KCC2 was sufficient to reverse the polarity of GABA mediated responses (Lee et al, 2005).

In immature neurons, prior to the expression of KCC2; NKCC1 (a widely expressed Na-K-Cl co-transporter), has been shown to be the main influence on chloride transport (Plotkin et al, 1997). NKCC1 tends towards neuronal accumulation of chloride, maintaining the high neonatal intracellular concentration of the cation (Schomberg et al, 2003). Its expression decreases reciprocally as KCC2 expression increases with maturation, except in dorsal root ganglia where it remains the predominant transporter. The situation appears to be further complicated, as it has been shown that hippocampal neurons initially express an inactive form of KCC2 which becomes activated during subsequent neuronal maturation. Studies in cultured hippocampal neurons suggest that this process involves endogenous protein tyrosine kinases and may be responsible for some of the apparent mismatch between the expression of KCC2 and functional changes $GABA_A$ receptors (Kelsch et al, 2001). However, recent work with embryonic cultured rat cortical neurons transfected to

over express human KCC2 at a developmental stage before endogenous upregulation of the transporter, has revealed that this is sufficient to end the depolarising period of GABA in these cells (Lee et al, 2005). This suggests that upregulation of KCC2 is not only necessary, as shown by knockout (Hubner et al, 2001) or blockade (Coull et al, 2003), but is in fact sufficient to alter the polarity of GABA signalling indicating that post translational steps are not rate limiting in cortical neurons. Therefore, the relationship between KCC2 levels and functional maturational changes may vary between different areas of the CNS. Beyond the direct functional effects of the 'switch' caused by KCC2 upregulation, recent reports in hippocampal neurons have suggested that the excitatory to inhibitory shift in GABAergic signalling selectively augments the expression of functional GABA synapses, but has no impact on glutamatergic synapses (Chudotovora et al, 2005).

1.8.3 Activity Dependence

The nature of the specific mechanisms responsible for driving the developmental 'switch' remain a contentious issue. Ganguly et al studied hippocampal neurons in culture, and reported that the developmental 'switch' was delayed by chronic blockade of $GABA_A$ receptors, and accelerated by increased $GABA_A$ receptor activation. In contrast, they found no effect from the blockade of glutamatergic transmission or action potentials. Furthermore, they were able to demonstrate that GABAergic activity modulated the mRNA levels of KCC2 (Ganguly et al, 2001). These findings were not reproduced by Ludwig et al in 2003, who found that neither neither neuronal spiking, nor glutamatergic and GABAergic transmission were required for the developmental expression of KCC2 in mouse hippocampal neurons in dissociated cultures or organotypic cultures (Ludwig et al, 2003). The view that

GABAergic activity was not an absolute requirement for the developmental upregulation of KCC2 gained further weight from an electrophysiological study in cultured rat midbrain neurons, showing that GABA was not required for the functional developmental switch of GABAergic responses from depolarising to hyperpolarizing (Titz et al, 2003). However, a further electrophysiological study in rat lateral superior olive (LSO), demonstrated that bilateral cochlear ablation before the onset of hearing appeared to disrupt the normal developmental change in chloride concentration seen in LSO neurons. Activation of GABAergic and glycinergic neurons before the onset of hearing (around P10-P12) induces neuronal depolarisation in the LSO. Therefore, these results appear to give support to the hypothesis that this depolarising afferent input is important in the development of the adult pattern of chloride homeostasis (Shibata et al, 2004). Hence, there remains a lack of consensus among the studies conducted *in vitro*. Jean-Xavier et al investigated the impact of thoracic spinal cord transection on the day of birth, and thus suppressing supraspinal influences, on the post-natal maturation of lumbar motoneurons. They found that neuronal responses at P4-7 were significantly more depolarising in the transected animals than in controls (Jean-Xavier et al, 2004 abst). Thus, influences from the brainstem may play an important role in setting the proper balance between excitation and inhibition in spinal networks. Leitch et al sought to investigate the upregulation of KCC2 *in vivo*, by using a sustained release preparation of bicuculline applied to the eye of newly hatched turtles, thus blocking GABAergic transmission during the period of normal upregulation. At 28 days post hatch, the retinae of the bicuculline treated turtles were found to have significantly less KCC2 immunoreactivity, and to display depolarising responses to GABA in a manner analogous to immature cells (Leitch et al, 2005). The factors driving the

developmental 'switch' in neonatal superficial dorsal horn neurons have not been fully investigated to the best of our knowledge, and will be addressed in this thesis.

1.8.4 Pathological Alterations

Once KCC2 has reached adult levels, and $GABA_A$ / glycine responses are hyperpolarizing, inhibitory synapses retain a degree of plasticity, and further changes are possible. Patch clamping after vagal axotomy in mature rats shows a disruption of chloride ion regulation, which is attributable to a decrease in KCC2 expression, and in turn leads to an excitatory response to GABA (Nabekura et al, 2002). Toyoda et al, confirmed this observation by performing a similar study on injured facial motoneurons, and go on to suggest that the GABAergic depolarisation may play a role in neuronal survival and regeneration (Toyoda et al, 2003). Coull and colleagues investigated the impact of peripheral nerve injury on GABAergic inhibition in lamina I neurons of the dorsal horn. In this case, the effect would need to be trans-synaptic as it would occur down stream from the site of the injury. They used immunoblotting on horizontal slices of superficial dorsal horn, ipsi and contra-lateral to peripheral nerve injury, as well as in naïve animals; and showed that the level of KCC2 on the ipsilateral side was about half that of the contralateral side with no change in the naïve animals. In order to confirm the role of KCC2, they patch clamped lamina I neurons after KCC2 knock-down and in the presence of bath applied [(dihydroindenyl)oxy]alkanoic acid (DIOA, KCC2 blocker); and compared these to the spinal neurons examined after peripheral nerve injury. Indeed, as in the case of the peripheral nerve injury dorsal horn neurons, 30% of cells showed an increase in Ca^{2+} in response to GABA (Coull et al, 2003).

Inflammatory pain models appear to have a different effect on KCC2 levels to that of neuronal injury. In a rat model of arthritis, KCC2 and KCC2 mRNA in the superficial dorsal horn were shown to be increased, with no change found in the deeper laminae. The change reached statistical significance by day 4 post-inflammation, and KCC2 levels returned to normal by day 10. These changes occurred bilaterally, but only reached statistical significance on the ipsilateral side (Morales-Aza et al, 2004). In inflammation, neuronal activity is usually increased without the confounding variable of neuronal injury, and therefore the mechanisms involved are likely to be distinct. It appears plausible that KCC2 increasing in response to an inflammatory insult is a protective physiological response, leading to increased inhibitory input, whereas KCC2 decrease following neuronal injury could be a pathological response to the damage.

Ischemic injury to the CNS results in loss of ionic homeostasis and the development of neuronal death. An in-vitro model of ischemic insult applied to mature hippocampal neurons, has shown that KCC2 is downregulated following oxygen-glucose deprivation (Galeffi et al, 2004). The $GABA_A$ agonist diazepam is known to be neuroprotective *in vivo*, when given early after a period of ischemia. The mechanism for this is not fully elucidated, but the same authors have shown that diazepam prevented the downregulation of KCC2 found in their model. KCC2 has also been studied in the hippocampus of human epileptic patients post-mortem. The expression pattern in non-epileptic patients was similar to that described in rodents. The epileptic patients had reduced expression of KCC2 in the areas of their hippocampus known to be epileptogenic foci (Munoz et al, 2004, abst). The

excitation generated by the hyperpolarizing action of GABA in these areas may be a contributory factor in seizure-genesis.

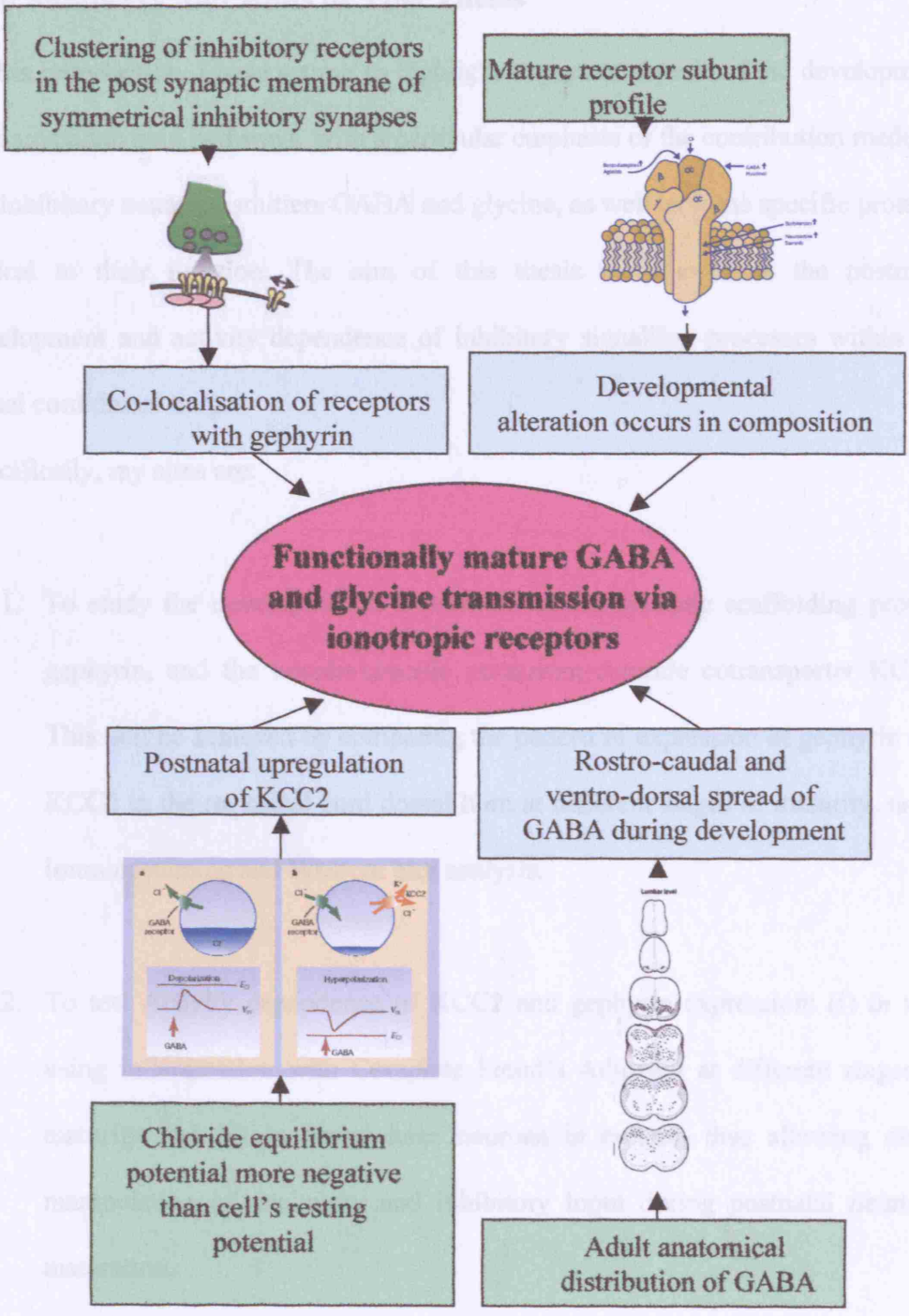
1.8.5 Conclusion

Thus, KCC2 expression has been suggested as a putative contributor to a number of pathological conditions, some of which have functional parallels with the immature nervous system. The ontological changes occurring in KCC2 in various parts of the CNS have been shown to alter the electrochemical gradient of developing neurons, and in turn to reverse the polarity of GABA and glycinergic signalling. This regulation of KCC2 appears to exhibit a degree of activity dependence, but the exact mechanism of this remains to be elucidated. The postnatal expression of KCC2 in the rat dorsal horn, as well as the factors regulating it will be addressed in this thesis.

1.9 The functional impact of GABA and glycinergic signalling

As discussed above, inhibitory transmission plays an important role in normal pain processing at the level of the spinal cord dorsal horn in the mature nervous system. Local blockade of glycinergic / GABAergic neurotransmission in the mature rat spinal cord, using intrathecally administered antagonists, causes hypersensitivity to mechanical stimulation (Loomis et al, 2001), in a manner analogous to the state found in neonatal rats. Similarly, it has been shown that intra-thecal administration of glycine / GABA is sufficient to prevent the development of neuropathic pain in animal models (Huang, 2000; Eaton et al, 1999). The tonic nature of at least a part of this spinal inhibition has recently been demonstrated by the intravenous administration of strychnine (glycine antagonist) and picrotoxin ($GABA_A$ antagonist) to naïve adult rats. This was shown to lead to C-Fos activation in several regions of

the spinal cord, indicating the presence of $GABA_A$ and glycinergic activity 'at rest' (Cronin et al, 2004). In view of the developmental changes discussed above, it could be speculated that the effects of intrathecally administered doses of GABA, glycine and their antagonists in neonatal rats may be different to those observed in adults. The effect of these compounds in the immature nervous system *in vivo* remains to be elucidated, and will be addressed in this thesis.



Developmental requirements for mature GABA & glycine transmission

for references, see text

1.10 Summary and Aims of This Thesis

In this introduction, I have set out to highlight important aspects of the development of mammalian pain pathways, with a particular emphasis of the contribution made by the inhibitory neurotransmitters GABA and glycine, as well as some specific proteins critical to their function. The aim of this thesis is to examine the postnatal development and activity dependence of inhibitory signalling processes within the spinal cord dorsal horn.

Specifically, my aims are:

1. To study the developmental expression of the synaptic scaffolding protein gephyrin, and the neuron-specific potassium-chloride cotransporter KCC2. This will be achieved by comparing the pattern of expression of gephyrin and KCC2 in the rat spinal cord dorsal horn at different stages of maturity, using immunostaining and Western blot analysis.

2. To test Activity dependence of KCC2 and gephyrin expression: (i) *in vivo* using inflammation with Complete Freud's Adjuvant at different stages of maturity and (ii) in dorsal horn neurons in culture, thus allowing direct manipulation of excitatory and inhibitory input during postnatal neuronal maturation.

3. To investigate the behavioural effect of intra-thecal injection of GABA, glycine and their antagonists in intact animals at various stages of maturity, in

order to test the hypothesis, *in vivo*, that GABA and glycine may have a quantitatively different effect at different ages.

4. To relate the findings to clinical practice by the use of a questionnaire examining clinicians' experience of using benzodiazepines in premature infants.

Chapter Two

Developmental Expression of KCC2 and Gephyrin in the superficial dorsal horn of the spinal cord

2.1 Introduction

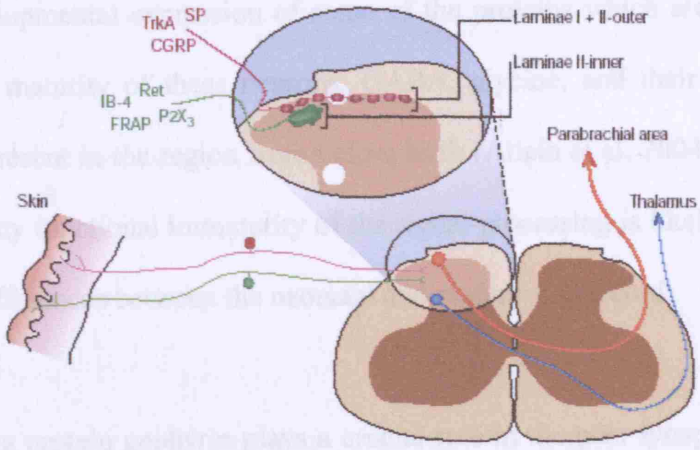
The superficial dorsal horn is a morphologically distinct region of the spinal cord that has been recognised as a separate entity for almost 200 years. It was first described by the anatomist Rolando in 1824, when he noticed the gelatinous appearance of the dorsal most region of the grey matter (Cervero & Iggo, 1980). As it contains the first synaptic relay of afferent pain fibres, it is important for the processing of signals relating to the transmission and modulation of pain. Many of the observed differences between neonatal pain processing and that of adult animals have their molecular basis in this region. Therefore, this chapter will examine the early postnatal expression of a number of proteins whose presence reflects an important functional role in the processing of pain information.

This will be compared with results from the tissue of mature animals, and interpreted in the light of immunostains known to reflect nuclear density and the presence of certain types of primary afferent fibres:

(i) NeuN is a recognised marker of neuronal cell nuclei. It therefore highlights the grey: white matter border, giving an idea of the grey: white matter ratio, as well as the relative nuclear density of the grey matter at various stages of development.

(ii) Calcitonin gene related peptide (CGRP) stains peptidergic terminals of C fibres and small diameter A fibres. Using the expression of these markers over the postnatal period, the maturation of peptidergic C fibre terminals in the superficial dorsal horn will be mapped.

(iii) Isolectin B4 (IB4) marks non-peptidergic C fibre terminals and therefore when used in a similar fashion to CGRP (above) will show their maturation in the postnatal dorsal horn.



CGRP and IB4 distribution in the context of mature pain pathways,

from Hunt & Mantyh, 2001

The changes in nuclear density demonstrated by NeuN provide a useful canvas for studying the relative expression of other proteins. The postnatal arrival of C fibres in the dorsal horn has been suggested to be of great functional importance in the maturation of nociceptive circuitry. The subsequent retraction of the A fibres to the deeper laminae (Shortland et al, 1990), the functional maturity of certain descending inhibitory mechanisms (Cervero & Plenderleith, 1985), as well as the reduction in dorsal horn neuron receptive fields (Fitzgerald, 1983) have all been linked to the arrival of C fibres. Thus mapping their distribution throughout development may yield important clues to developmental signals occurring in the region.

Much of the information processing in the dorsal horn is dependent on inhibitory interneurons, which have their effect via the transmitters GABA and glycine. These interneurons mature later than projection neurons (Bicknell & Beal, 1984). Physiological studies suggest that some inhibitory mechanisms attributed to interneuronal activity appear to be absent in early development (Fitzgerald, 2005;

Pattinson & Fitzgerald, 2004). This chapter will therefore go on to explore the postnatal developmental expression of some of the proteins which are important to the functional maturity of these neurons. GABA, glycine, and their receptors are known to be present in the region from before birth (Allain et al, 2004; Watanabe et al, 1995), so any functional immaturity of the signal processing is likely to be due to more subtle differences between the neonatal and mature spinal cord.

The scaffolding protein gephyrin plays a crucial role in the post synaptic membrane of inhibitory synapses by forming clusters of receptors and thus increasing their post synaptic density at these symmetrical synapses (Sassoe-Pognetto & Fritschy, 2000). It is therefore conceivable that differences in gephyrin levels may have a quantitative functional impact. Here the topographical expression of gephyrin was studied by immunostaining, and quantified by western blot analysis.

The effects of GABA and glycinergic transmission have also been noted to be qualitatively different in early postnatal life, in certain areas of the CNS (Obata et al, 1978). This is attributable to differences in the electrochemical gradient of chloride ions across the cell membrane. Inhibitory signalling through $GABA_A$ receptors is dependent on active extrusion of chloride from post-synaptic neurons, and requires the presence of cation-chloride co-transporter molecules. KCC2 is a neuron specific cation-chloride transporter, whose upregulation has been shown to be causally related to the switch from excitatory to inhibitory properties of GABA receptors (Rivera et al, 1999). Thus, levels of KCC2 in the neonatal dorsal horn are likely to be of crucial functional importance, as they have the potential to reverse the polarity of

GABA and glycinergic transmission. In this chapter, the postnatal distribution of KCC2 in the spinal cord and its quantitative expression will be investigated.

Many maturational changes in the developing spinal cord are known to be activity dependent (Ren et al, 2004; Waldenstrom et al, 2003; Andrews & Fitzgerald, 1999; Beggs et al, 2002), with reduced or altered sensory input leading to developmental anomalies lasting a lifetime. The expression of gephyrin and KCC2 were therefore examined in the presence of persistent inflammation, produced by intra-plantar injection of complete Freund's adjuvant. This was achieved by the comparison of the ipsilateral and contralateral dorsal horn with that of a naïve animal of the same age, using western blot analysis.

In summary, this chapter sets out to investigate the postnatal expression of functionally important proteins in the superficial dorsal horn of rats. The patterns of expression are examined immunohistochemically, before a quantitative measurement using Western blot analysis. Finally, the activity dependence of postnatal changes is examined using an *in vivo* model of inflammation.

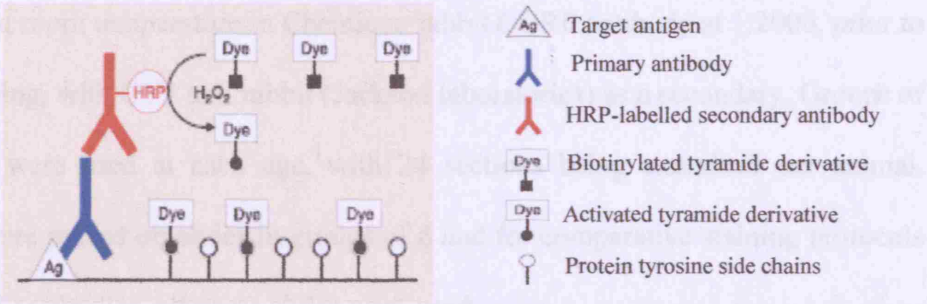
2.2 Materials and Methods

Sprague Dawley rats of both sexes were used; these were bred by the Central Animal Facility, University College London. They were kept in artificial lighting on a 12:12h light cycle and the temperature was kept constant at 21°C. Food and water were given *ad libitum* and all procedures were carried out in accordance with the United Kingdom Animal Procedure Act 1986. Adult rats and postnatal day (P) 3,10, and 21 animals were studied. P3 and P10 rat pups were housed with their mother and littermates. P21 and adult rats were housed independently in groups of 4-6 animals.

2.2.1 Gephyrin Immunohistochemistry

The animals were terminally anaesthetised with an intra peritoneal, lethal solution of sodium pentobarbital (Euthatal). Their spinal cords were then removed by dorsal lamminectomy, mounted in Tissue-Tek OCT compound (Sakura Finetek) and transverse sections of 20 microns were cut using a cryostat through the lower lumbar (L4, L5) spinal cord. These were then mounted on to gelatinised slides before being fixed by immersion in a fixative solution containing 2% w/v paraformaldehyde in 0.1M phosphate buffer (PB) for 90 seconds and twice washed in 0.1M PB. Following this the sections were blocked in a solution of 0.1M PBS, containing 5% normal goat serum (Vector), and 0.25% fish gelatin (Sigma), for 2 hours at room temperature.

The tyramide Signal Amplification (TSA) technique or 'blast' protocol was employed (TSA biotin system, Perkin Elmer). This enzyme mediated detection method uses the catalytic activity of horseradish peroxidase to generate high density labelling of a target protein.



Schematic diagram of the TSA detection method

Biotin labelled tyramide is bound onto previously blocked tissue following the primary antibody application, as a result of a rapid reaction numerous biotin labels are left close to the protein of interest. Thus, TSA allows the ultra sensitive detection of low abundance targets.

Sections were incubated in Mab 7a, a commercially available, mouse monoclonal antibody against gephyrin (Alexis Biochemicals) at 1:1000, made up in 0.05M tris-saline with 0.3% triton X-100 (TTBS), and 5% normal goat serum, overnight at 6°C. The slides were then washed 3 times for 10 minutes in 0.1M PB before being incubated for 90 minutes at room temperature in biotinylated goat anti mouse (Vector) at 1:400. Following this, they were washed again as above, and incubated in Vectastain Elite ABC amplification kit solution for 30 minutes. After a further wash the slides were placed in biotinylated tyramide solution (Perkin Elmer TSA biotin system) for 7 minutes, before washing once again. Lastly, the slides were incubated in Fluoresceine Avidin C (FITC), Vector, 1:600 for 2 hours. Control sections were treated in the same way, except that the primary antibody was omitted. Finally, the sections were cover slipped in Gel Mount (Sigma). For the double staining

experiment with calcitonin gene related peptide (CGRP), the slides were incubated overnight at room temperature in Chemicon rabbit CGRP antibody at 1:2000, prior to cover slipping, with CY3 anti rabbit (Jackson laboratories) as a secondary. Groups of 4 animals were used at each age, with 24 sections being examined per animal. Sections were placed on slides in groups of 6 and for comparative staining protocols involving 2 antibodies, alternate slides were used.

2.2.2. KCC2 Imuunohistochemistry

Rats of the same ages used above were terminally anaesthetised as before. They were then perfused transcardially first with heparinised saline, followed by a fixative solution containing 4% paraformaldehyde in 0.1M phosphate buffer. The lumbar enlargement of their spinal cords were then removed, and post-fixed in the same solution for 2 hours at room temperature. These cord segments were then cryo-protected by overnight immersion in a 30% sucrose phosphate buffer at 6°C. Following this they were mounted in Tissue-Tek OCT compound (Sakura Finetek) and transverse sections of 20 microns were cut, using a Leika cryostat, through the lower lumbar (L4, L5) spinal cord. These were thaw-mounted on to gelatinised slides, and then the sections were blocked in a solution of 0.1M PBS, containing 5% normal goat serum (Vector), and 0.25% fish gelatin (Sigma), for 2 hours at room temperature. Next they were incubated in a rabbit polyclonal antibody raised against KCC2 (Chemicon) at 1:2000, made in 0.05M tris-saline with 0.3% triton X-100 (TTBS), and 5% normal goat serum, overnight at 6°C. The indirect tyramide amplification procedure was carried out as above, and once again, control sections were treated in the same way except for omission of the primary antibody. Sections

were cover slipped as before. Groups of 4 animals were used at each age, with 24 sections being examined per animal. Sections were placed on slides in groups of 6 and for comparative staining protocols involving 2 antibodies, alternate slides were used.

2.2.3 Other Immunohistochemical Experiments

Rats of the same ages were perfused trans-cadially as above. The following antibodies were used to illustrate the developmental changes occurring in the superficial dorsal horn:

- IB4 (Fluorescein Griffonia Simplifolia Lectin I, Vector) incubated at 1:100 for 16 hours; as a marker of non-peptidergic C fibre terminals;
- Anti-CGRP (calcitonin gene related peptide, Chemicon) used at 1:2000 for 22 hours followed by a goat anti-rabbit secondary antibody (1:500, Alexa-594); as a marker of peptidergic C fibres;
- Anti-NeuN (Chemicon) used at 1:500, then biotinylated before addition of the secondary antibody (FITC 1:600, 2 hours, Vector); as a marker of nuclear density.

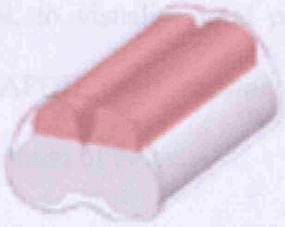
Washes and cover-slipping were carried out as before.

2.2.4 Microscopy

Sections were visualised using a fluorescent microscope (Eclipse E-800, Nikon with Nikon objectives). Electronic images were captured by a CCD camera (CoolSnapsC5, Roper Scientific Photometric) and MCID imaging software.

2.2.5 Western Blot Analysis

Neonatal rat pups (P3, P10, P21) and adult rats were terminally anaesthetised with a lethal solution of sodium pentobarbital (Euthatal), the L3 – L6 segment of their lumbar spinal cord was harvested, and the dorsal halves (as shown in pink) were isolated before being snap frozen in liquid nitrogen and stored at –80 C.



Spinal cord L3-L6 segments were divided in the plane shown, to separate dorsal and ventral horns.

Samples of spinal cord from individual animals (n= 6 at each age) were homogenised in RIPA buffer containing a cocktail of proteinases and phosphatase inhibitors (1% NP-40, 20mM Hepes pH 7.4, 100mM NaCl, 100 mM NaF, 1mM NaVO4, 5 mM EDTA, leupeptin 10 microg/ml, and pepstatin 1 microgram/ml; Sigma), on ice. Homogenates were then left for 2 hours on ice, and centrifuged (12, 000 g, 15 mins). Supernatants were collected and total protein concentration was titrated using a BCA kit (Pierce, Rockford. IL). The samples were then twice normalised to contain an equivalent amount of total protein.

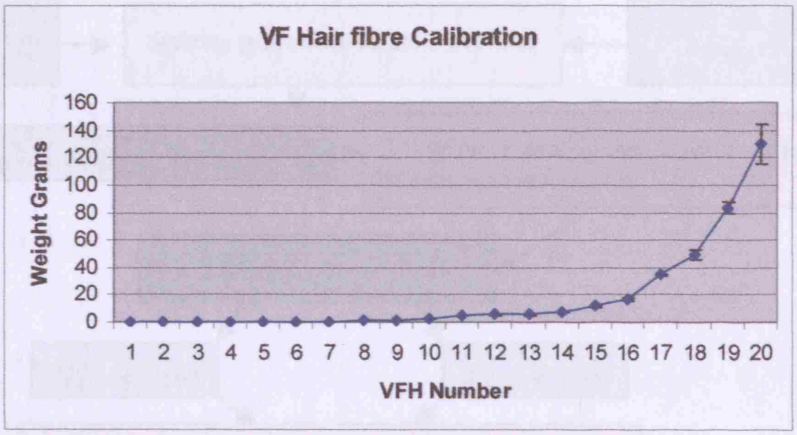
Protein samples were separated on a SDS-PAGE gel (10% Bio-Rad), and transferred to PVDF filters. The blots were blocked with 5% skimmed milk for 1 hour, and incubated with Mab 7a, a commercially available mouse monoclonal antibody against gephyrin (Alexis Biochemicals) at 1: 1500, or KCC2 (Chemicon) at 1:2000 overnight at 4°C. Following 10 washes of 5 minutes each in PBS with 0.1% Tween-

20, the blots were incubated in HRP-conjugated secondary antibody (1:2000) for 45 minutes at room temperature. They were then washed a further 10 times in PBS-Tween and developed in ECL solution (Amersham) for 5-10 minutes. The blots were then incubated in stripping buffer for 20 minutes and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:750, Chemicon) overnight at 4 C as a loading control. Coomassie brilliant blue dye (R-250) was used as an alternative loading control, to visualise total protein, in later experiments as developmental changes in GAPDH itself became apparent. Each individual Western blot was repeated a minimum of twice.

2.2.6 Hind-paw Inflammation Using Complete Freund's Adjuvant

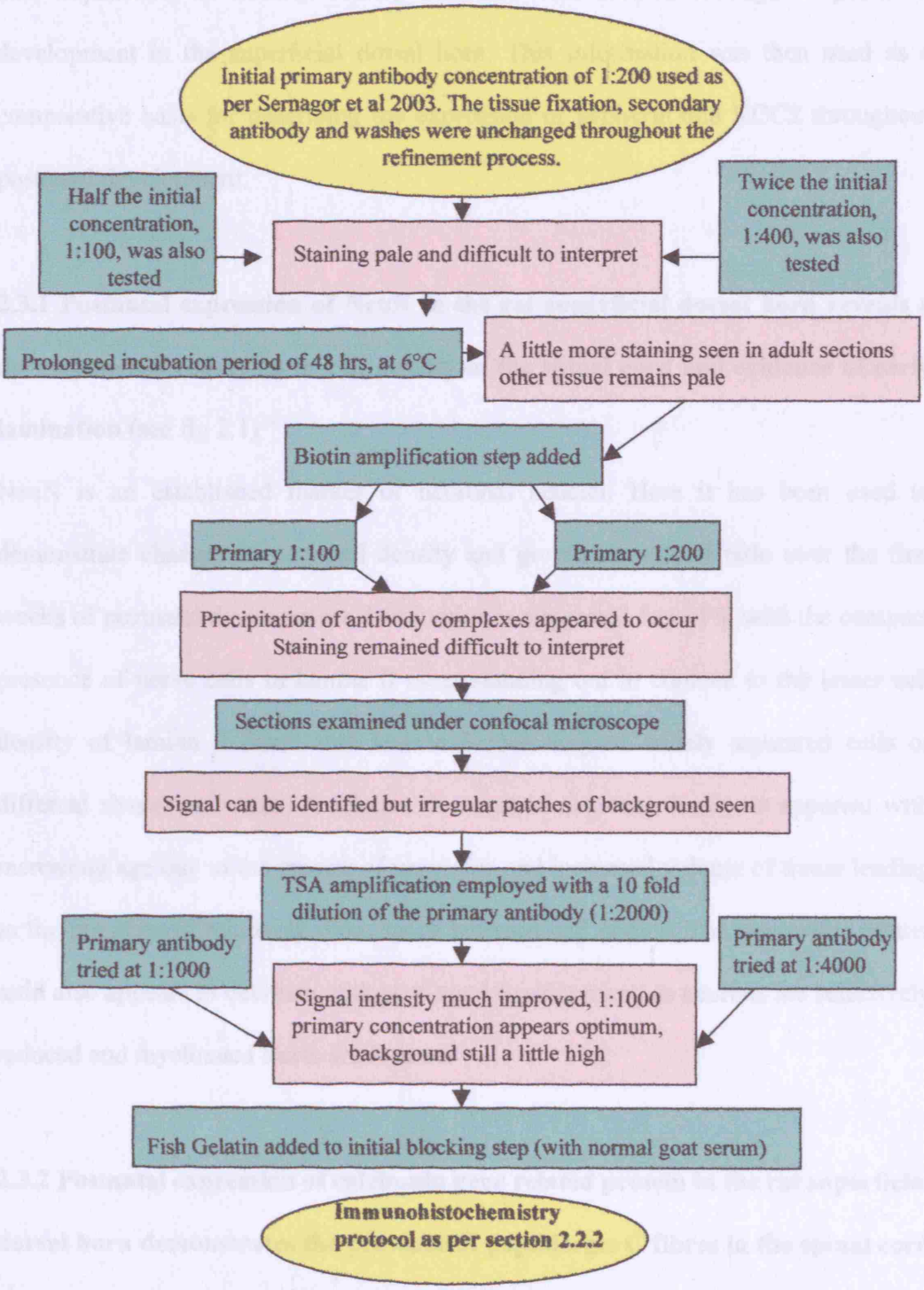
Protein quantification by Western blot analysis was repeated using animals in which the hind-paw had been inflamed three days earlier, by intra-plantar injection of Complete Freund's Adjuvant (CFA). Rats aged P0, P7 and P18 (n=6 at each age) were anaesthetised with 4% halothane in oxygen in a Perspex box, before receiving an injection of CFA (Sigma Aldrich; 1mg *Mycobacterium Tuberculosis* in 0.85 ml mineral oil and 0.15 ml mannide mono-oleate diluted 1:1 with normal saline) into the left hind-paw, using a 30 gauge sterile needle and a calibrated Hamilton syringe. The volumes used were adjusted according to the age of the rat pups, with P0 animals receiving 3µl, P7 animals 10µl, and P18 animals 25µl. Baseline measurements of paw diameter and mechanical withdrawal threshold were recorded prior to the procedure. Paw diameter was measured at the midpoint of the hind-paw using a calibrated calliper across the dorsal to plantar surface. The inflammation was defined by the percentage increase in paw diameter three days post injection (at P3, P10 & P21). Mechanical thresholds were assessed by applying Von Frey Hairs (nylon

monofilaments of increasing thickness) to the plantar surface of the hind-paw, until the force produced was sufficient to produce a withdrawal reflex on three out of five applications (60% threshold). Von Frey Hair testing was also repeated three days after the injection of CFA and the results were compared.



Calibration of von Frey hairs prior to use.

Following this, the animals were terminally anaesthetised with a lethal solution of sodium pentobarbital (Euthatal), and the L3 – L6 segment of their lumbar spinal cord was harvested, and prepared for Western blot analysis as previously described. The samples were analysed with previously harvested naïve tissue of the same ages (n=6 at each age).



Flow diagram of refinements made to immunohistochemistry protocol with KCC2 used as an example

2.3 Results

The expression of NeuN, CGRP, and IB4 was studied throughout postnatal development in the superficial dorsal horn. This information was then used as a comparative basis for describing the expression of gephyrin and KCC2 throughout postnatal development.

2.3.1 Postnatal expression of NeuN in the rat superficial dorsal horn reveals a developmental reduction in cell density in the spinal cord and evidence of early lamination (see fig 2.1)

NeuN is an established marker of neuronal neuclei. Here it has been used to demonstrate changes in neuronal density and grey:white matter ratio over the first weeks of postnatal development. Lamination is suggested from P3, with the compact presence of nerve cells in lamina II outer standing out in contrast to the lesser cell density of lamina II inner, and lamina I consisting of widely separated cells of different shapes and sizes. A relative decrease in neuronal density is apparent with increasing age due to the growth of processes and increased volume of tissue leading to the presence of relatively more space between cell bodies. The grey:white matter ratio also appears to decrease with post-natal development, as neurons are selectively reduced and myelinated tracts are formed.

2.3.2 Postnatal expression of calcitonin gene related protein in the rat superficial dorsal horn demonstrates the presence of peptidergic C fibres in the spinal cord from birth (see fig 2.2)

Calcitonin gene related protein is a neuropeptide used as a marker of peptidergic C fibre terminals. This subgroup are known to represent the majority of all C fibres

around the time of birth, as the other group (IB4+ve) mature postnatally in rat spinal cord (Bennett, 1996). CGRP+ve C fibre terminals are apparent from P3, but appear relatively superficial, located around lamina I & II outer. They seem to gradually spread to lamina II inner and form a wider band of greater intensity, with lateral exentuation at P10 and P21. The band of CGRP-like immunofluorescence occupied the uppermost 10% of the dorsal horn only at P3, extending to 17% at P10 and 21% at P21.

2.3.3 Postnatal expression of isolectin B4 in the rat superficial dorsal horn reveals the later arrival of non-peptidergic C fibres (see fig 2.3)

Here, IB4 immunofluorescence has been used to label non-peptidergic C fibre terminals. Consistent with their relatively late maturation, these are barely detectable at P3, however in mature cord a clear band of intensity is seen in the region of lamina II inner. This band begins to form at P10, becoming denser by P21.

2.3.4 The 93 kD scaffolding protein gephyrin is postnatally upregulated in the superficial laminae of the rat dorsal horn

The expression of gephyrin in the dorsal horn was investigated both qualitatively and quantitatively using a combination of immunohistochemistry and western blot analysis.

2.3.4.1 Gephyrin Immunohistochemistry (see fig 2.4)

Gephyrin staining was located principally on the cell membranes, corresponding to cell surface clusters. Very little gephyrin immunofluorescence is seen in the dorsal horn at P3. By the age of P10, staining is clearly seen in the deeper laminae, but this

remains somewhat diffuse and has failed to reach superficial areas. At P21 a concentrated band of fluorescence has begun to form but only in the region of lamina III, with some punctate expression visible in lamina I, and relatively little staining seen in lamina II. In the adult animal, this band becomes denser and appears to extend from lamina III outer to lamina II and into lamina I.

2.3.4.2. Double label immunohistochemistry (see figs 2.5 & 2.6)

In order to further qualify the apparent postnatal rise up the dorsal horn of gephyrin expression, a double labelling experiment was conducted with an antibody raised against CGRP (shown to form a band in laminae I & II, fig 2.2; Cervero et al, 1989). Correspondingly, at P10, a clear gap is seen between the bands of expression of CGRP and gephyrin, whereas in the adult cord co-localisation is obvious in lamina II.

2.3.4.3 Western blot analysis (see fig 2.7)

Although there is a clear post-natal regulation of the pattern of gephyrin expression in the dorsal horn, the quantitative expression remained to be elucidated. Therefore, western blot analysis was employed to compare the relative quantities of protein in the dorsal segment of the spinal cord at each age. Initial experiments were conducted with the house-keeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, however, it became apparent that GAPDH itself appears to undergo upregulation during early postnatal development, and coumassie blue dye was therefore used in preference as a loading control. Expression of gephyrin was shown to be significantly lower at P3 and P10 than in older animals ($n=6$, $P<0.05$, ANOVA). The upregulation of protein levels at P21 coincides with the appearance of

the dense band of staining in the superficial dorsal horn seen in the immunofluorecence studies.

2.3.5 The 140 kD cation-chloride co-transporter KCC2 is postnatally upregulated in the superficial laminae of the rat dorsal horn

The expression of KCC2 in the dorsal horn was investigated both qualitatively and quantitatively again using a combination of immunohistochemistry and western blot analysis.

2.3.5.1 KCC2 immunohistochemistry (see figs 2.8 & 2.9)

Some KCC2 immunofluorescence was apparent throughout the spinal cord grey matter at all ages, but the pattern and intensity changed greatly with age. Expression was located principally on the cell membrane. Although very little KCC2 staining is seen in the dorsal horn at P3, it is present ventrally in areas associated with motor function. At P10, a thin, superficial band of expression can be seen in laminae I and II outer, as well as some staining on dendritic fibres emerging medially between the dorsal horns. By P21 the band is wider, encompassing laminae I, II and III outer. At this stage some expression also becomes apparent in a lateral area corresponding with the lateral spinal nucleus. In the adult rat cord, the band remains dense and wide; the expression in the lateral spinal nucleus persists, although the staining of medial fibres emerging from the dorsal horn is now less prominent.

2.3.5.2 Western blot analysis (see fig 2.10)

The changing nature of KCC2 expression in the postnatal rat dorsal horn was quantified by western blot analysis of the dorsal half of the spinal cord. Levels of KCC2 were extremely low at P3 and a significant upregulation was shown between P3 and P10 ($n=6$, $P<0.05$, ANOVA), with a smaller increase, which failed to reach statistical significance occurring between P10 and P21. Levels in adult animals remained similar to those at P21.

2.3.6 Intra-plantar injection of complete Freund's adjuvant (CFA) produces measurable inflammation of the hind-paw at all ages, but only causes mechanical hyperalgesia from the age of P10 upwards (see figs 2.11, 2.12 & 2.13)

In order to assess the impact of an inflammatory lesion at various stages of development on the expression of gephyrin and KCC2, rat pups were given single intra-plantar injections of CFA at P0, P7 & P18. Paw thickness was found to be significantly increased 3 days later at P3, P10 & P21 ($n=6$, $P<0.05$, ANOVA), reflecting the presence of inflammation. However, as previously reported (Walker et al, 2003; Alvares et al, 2000) CFA failed to produce statistically significant mechanical hyperalgesia in P3 animals injected at P0 ($n=6$). Mechanical withdrawal thresholds, as measured by Von Frey hair testing, were significantly lower at P10 & P21 following CFA injection ($n=6$, $P<0.05$, ANOVA).

2.3.7 CFA mediated inflammation does not alter the postnatal expression of gephyrin (see figs 2.14, 2.15 & 2.16)

Western blot analysis was used to compare expression of gephyrin in the dorsal segment of the spinal cord ipsilateral and contralateral to plantar injection of CFA;

with that of naïve animals. Experiments were conducted at P3, P10 & P21, following injections of CFA 3 days earlier at P0, P7 & P18. In the case of gephyrin, no differences were shown between ipsilateral, contralateral or naïve tissue, at any of the three ages examined (n=6). It therefore appears that peripheral inflammation does not affect the developmental expression of gephyrin.

2.3.8 CFA mediated inflammation affects the postnatal expression of KCC2 from the age of P10 onwards (see figs 2.17, 2.18 & 2.19)

Western blot analysis was used to investigate the impact of CFA mediated inflammation on the expression of KCC2 at various developmental stages, in the same way as described above.

2.3.8.1 CFA mediated inflammation at P0 does not affect the expression of KCC2 at P3.

There was no significant difference in the expression of KCC2 in the dorsal horn following CFA mediated inflammation either at P3 ipsilateral or contralateral to the injury (n=6).

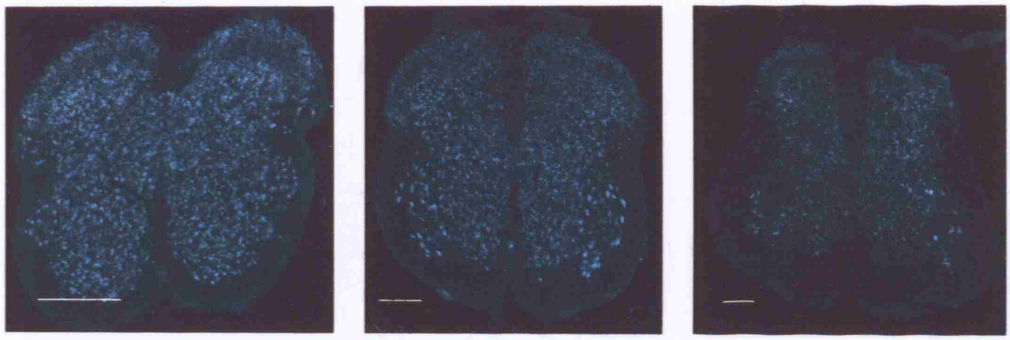
2.3.8.2 CFA mediated inflammation at P7 causes a significant ipsilateral increase in KCC2 expression in the rat dorsal horn at P10

By the age of P10, animals treated with intra-plantar CFA 3 days earlier had a significant increase in KCC2 in the dorsal horn, on the same side as the injury (n=6, $P<0.05$, ANOVA). No increase was found on the contralateral side, where levels were indistinguishable from those of naïve animals.

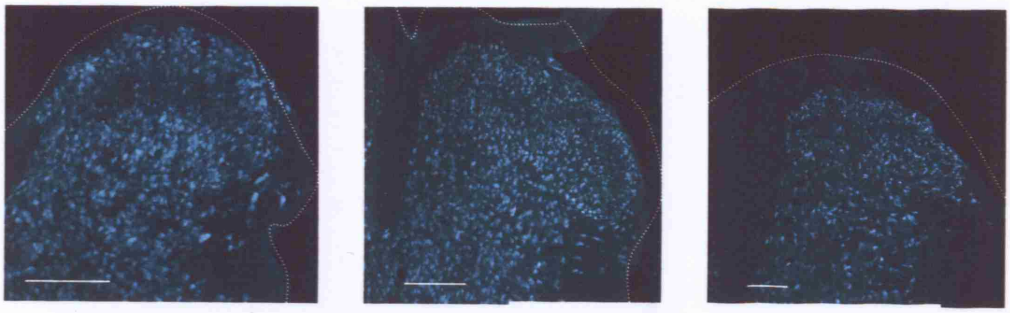
2.3.8.3 CFA mediated inflammation at P18 causes a bilateral increase in KCC2 expression in the rat dorsal horn at P21

In mature rats (P21), intra-plantar injection of CFA causes a significant increase in KCC2 in the ipsilateral dorsal horn ($n=6$, $P<0.05$, ANOVA), as well as an increase on the contralateral side, which fails to achieve statistical significance.

NeuN Staining of the Developing Rat Lumbar Spinal Cord



P3 P10 P21



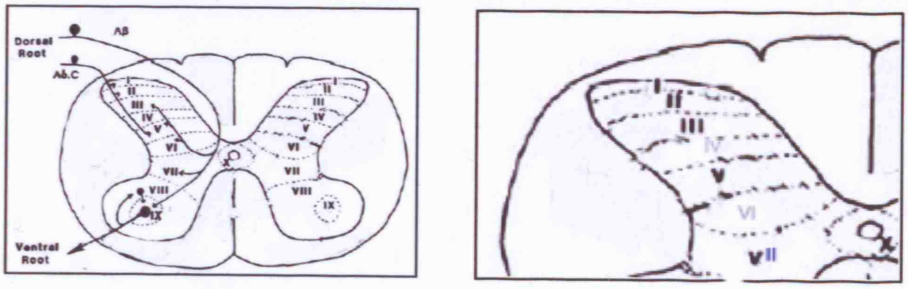
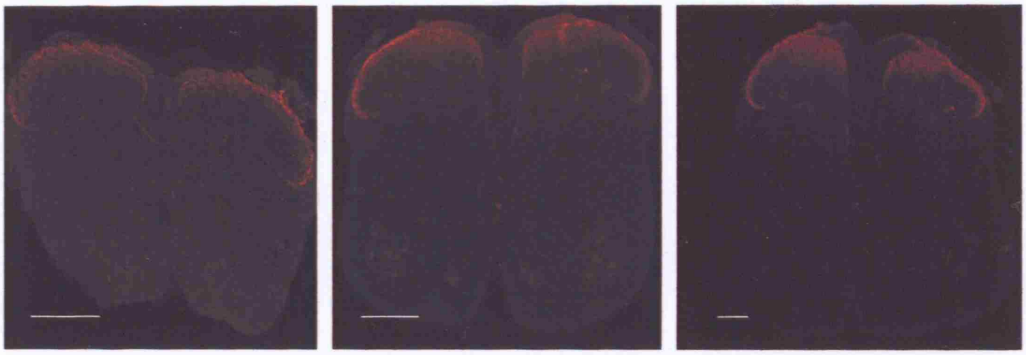


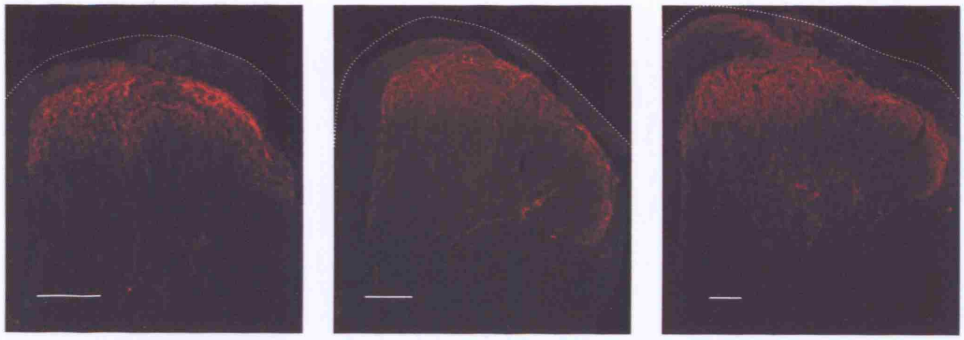
Fig 2.1

NeuN, a marker of neuronal neuclei, has been used to show nuclear distribution at various stages of postnatal development. Neuronal lamination is established by P3, and shows little change post-natally. The compact presence of nerve cells in lamina II outer stands out in contrast to the lesser density in lamina II inner. The grey:white matter ratio can be seen to alter with increasing age. Scale bars in whole cord images 200μm, dorsal horn only 100μm.

CGRP Staining of the Developing Rat Lumbar Spinal Cord



P3 P10 P21



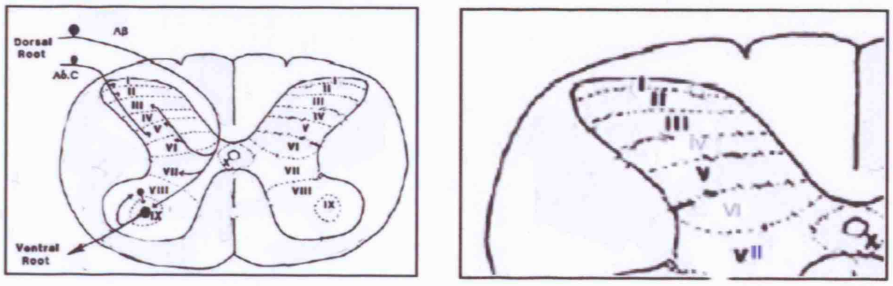


Fig 2.2

Calcitonin gene related peptide (CGRP) antibody has been used to label peptidergic C fibre terminals at various stages of postnatal development. The terminals are apparent from P3 but appear more superficial, located around lamina I & II outer. They seam to gradually descend to lamina II inner and form a band of greater width and intensity at P10 & P21.Scale bars in whole cord images 200μm, dorsal horn only 100μm.

IB4 Staining of the Developing Rat Lumbar Spinal Cord

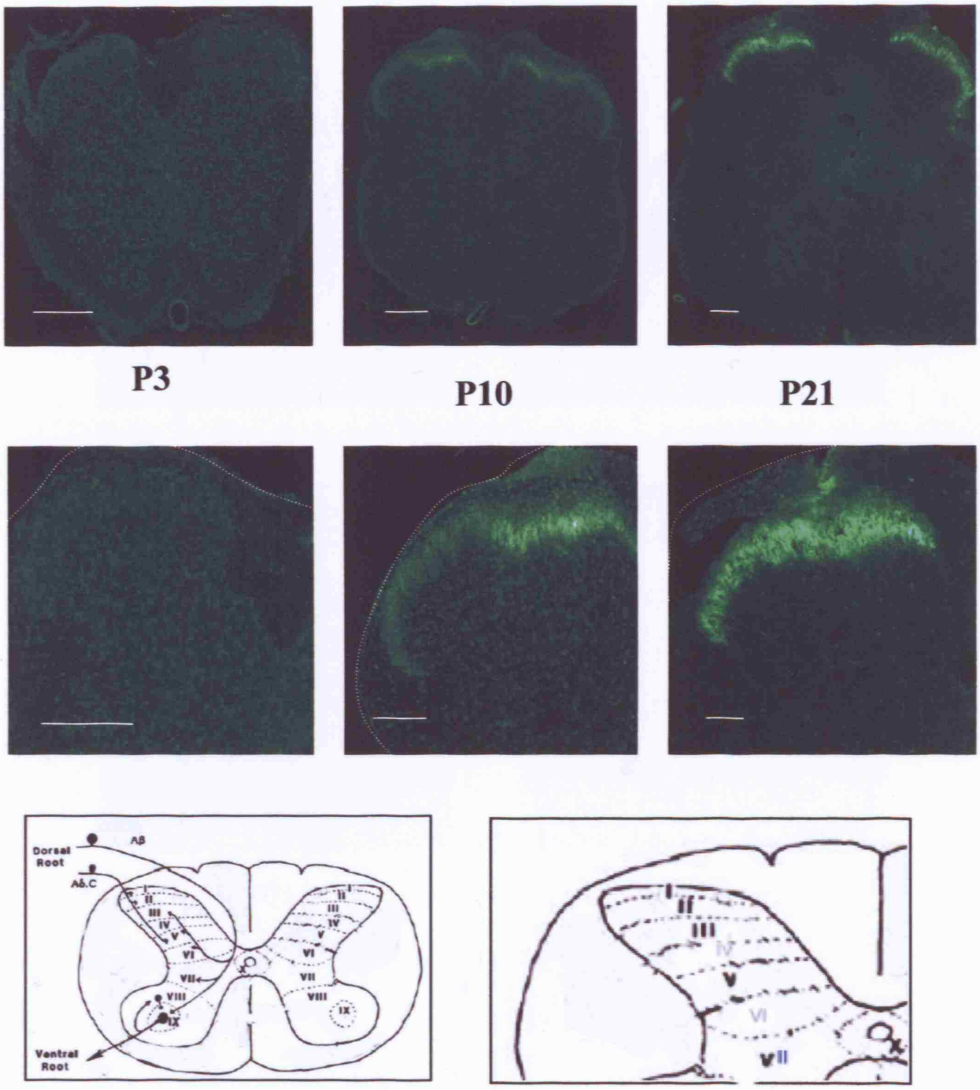


Fig 2.3

IB4 immunofluorescene has been used to label non-peptidergic C fibres at various stages of postnatal development. In mature cord, these are clearly seen in lamina II inner. There is no detectable staining in the dorsal horn at P3, but a distinct band forms by P10, which intensifies by P21. Scale bars in whole cord images 200μm, dorsal horn only 100μm.

Developmental Expression of Gephyrin in Rat Dorsal Horn

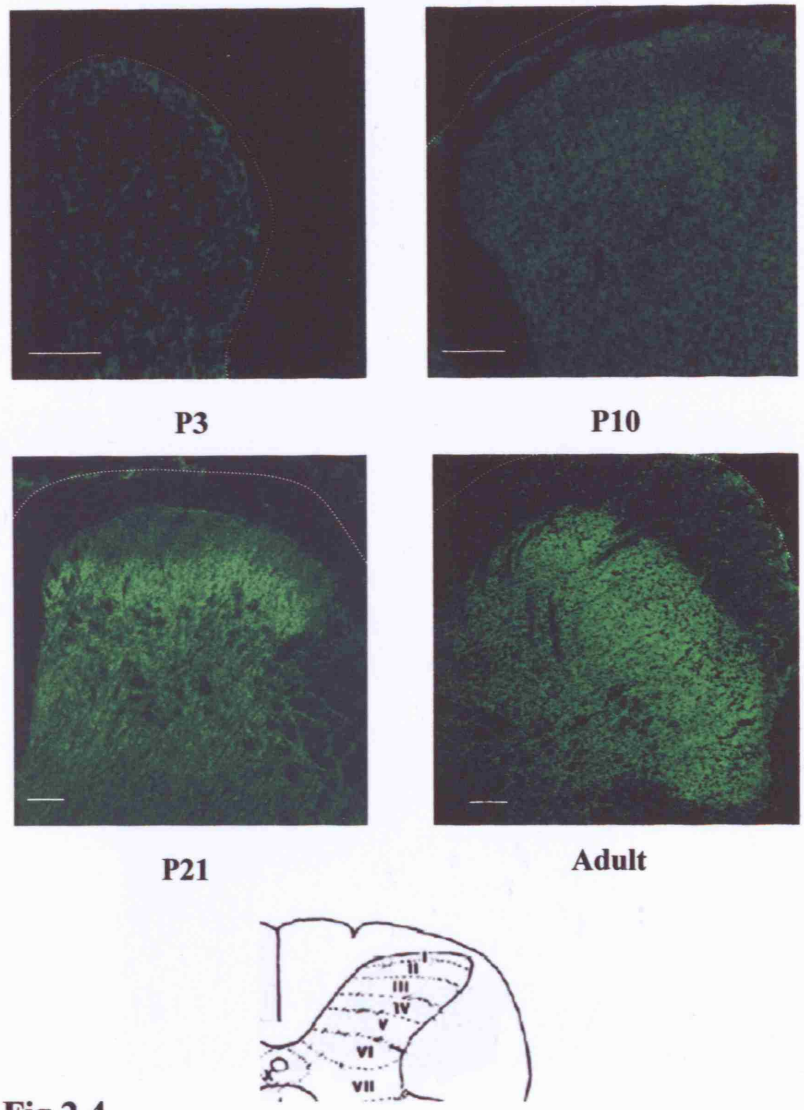
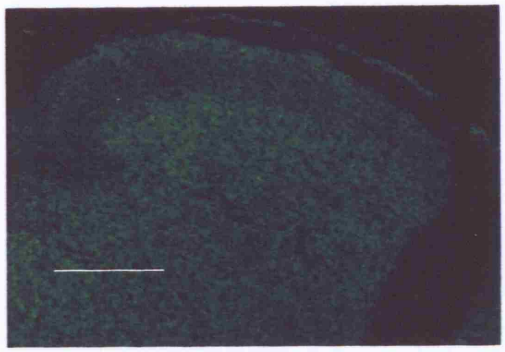


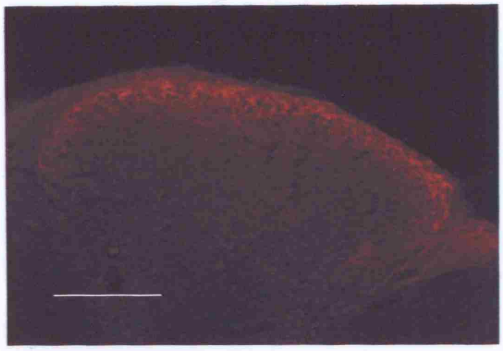
Fig 2.4

Developmental expression of gephyrin in the dorsal horn of rats. Little fluorescence is seen at P3, by P10 expression is rising up the dorsal horn. It begins to form a discreet 'band' at P21, which becomes more distinct in the adult animal.
Scale bars 100 μm. Diagram of laminae is shown for orientation.

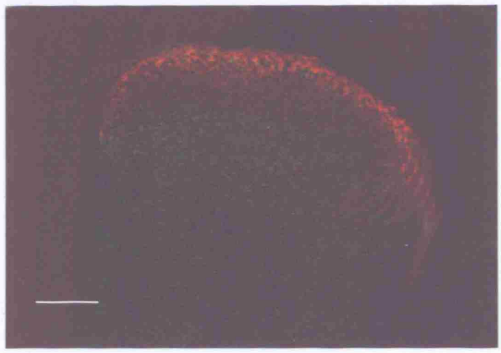
Gephyrin and CGRP Expression in P10 Rat Dorsal Horn



Gephyrin



CGRP

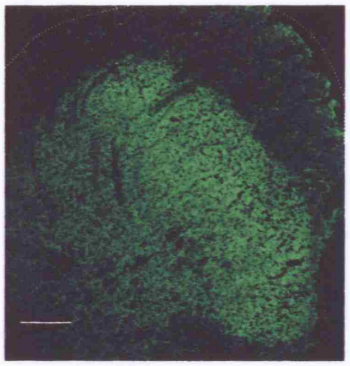


Gephyrin and CGRP

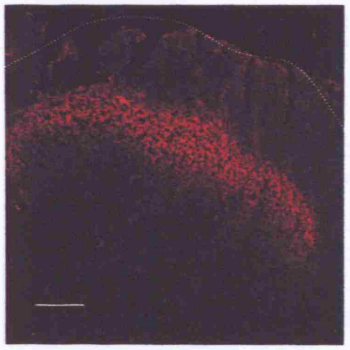
Fig 2.5

Expression of gephyrin (Green) and CGRP (red) in the dorsal horn of P10 rat pups. The 'bands' of expression in the dorsal horn of P10 rats are distinct from each other.
Scale bars 100μm

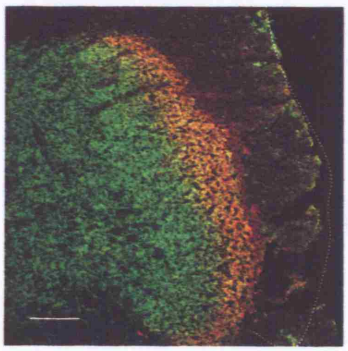
Gephyrin and CGRP Expression in Adult Rat Dorsal Horn



Gephyrin



CGRP



Gephyrin & CGRP

Fig 2.6
Expression of gephyrin and CGRP in the dorsal horn of adult rats. Overlap is shown between the 'bands' of CGRP and that of gephyrin in the dorsal horn. Scale bars 100 μm

Western Blot Analysis of the Postnatal Expression of Gephyrin

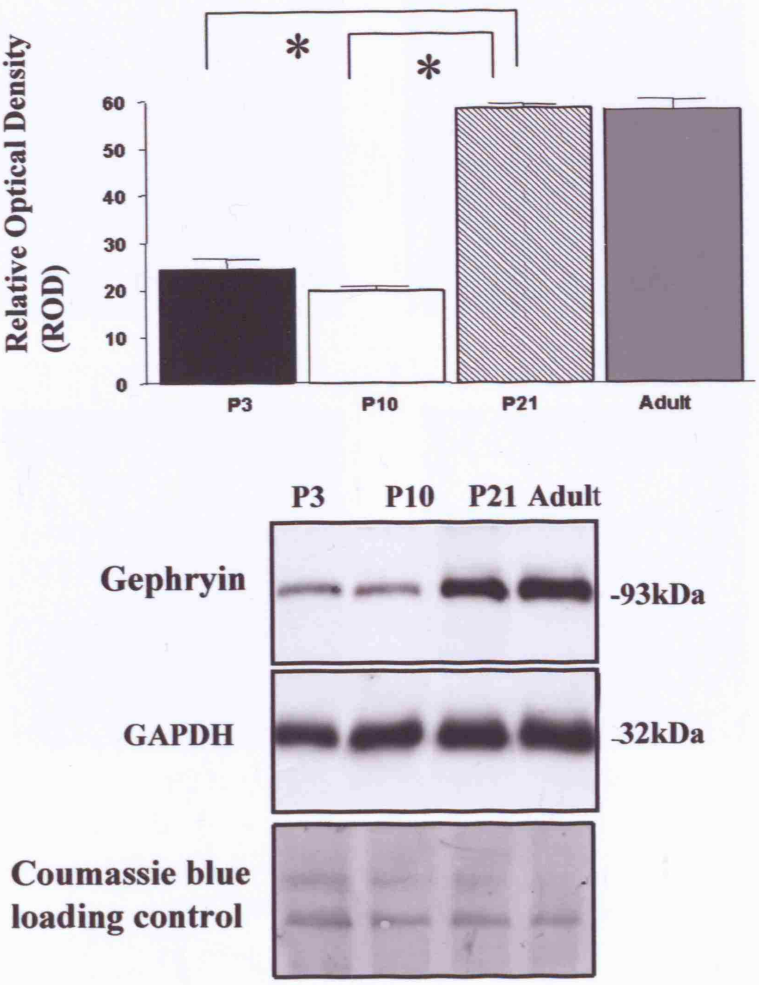


Fig 2.7
Western blot analysis demonstrating the post-natal up-regulation of Gephyrin. n=6 at all ages.
* $P<0.05$ (ANOVA)

Developmental Expression of KCC2 in Rat Dorsal Horn.

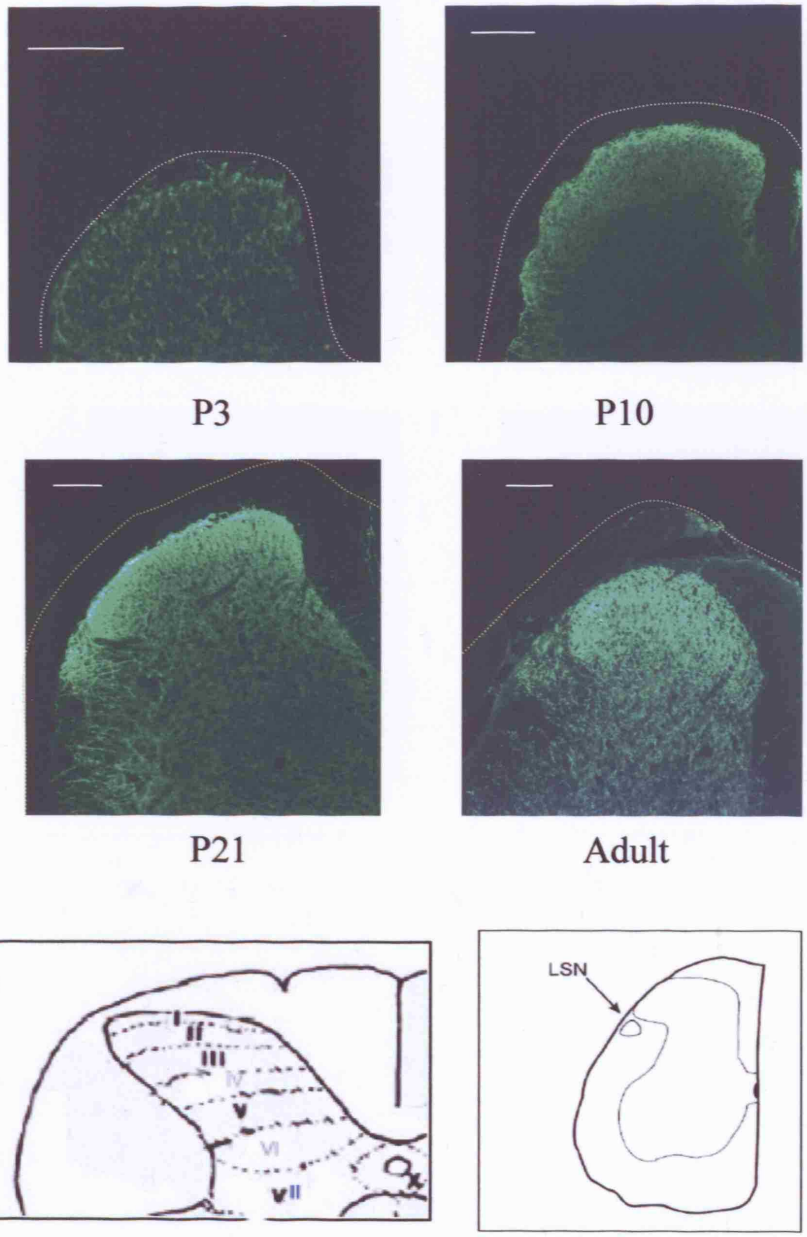


Figure 2.8
These close up views show the formation of the 'band' of expression of KCC2 in the mature rat superficial dorsal horn. Scale bars 100 μm. Map of laminae and the lateral spinal nucleus shown for orientation.

Developmental Expression of KCC2 in Rat Spinal Cord

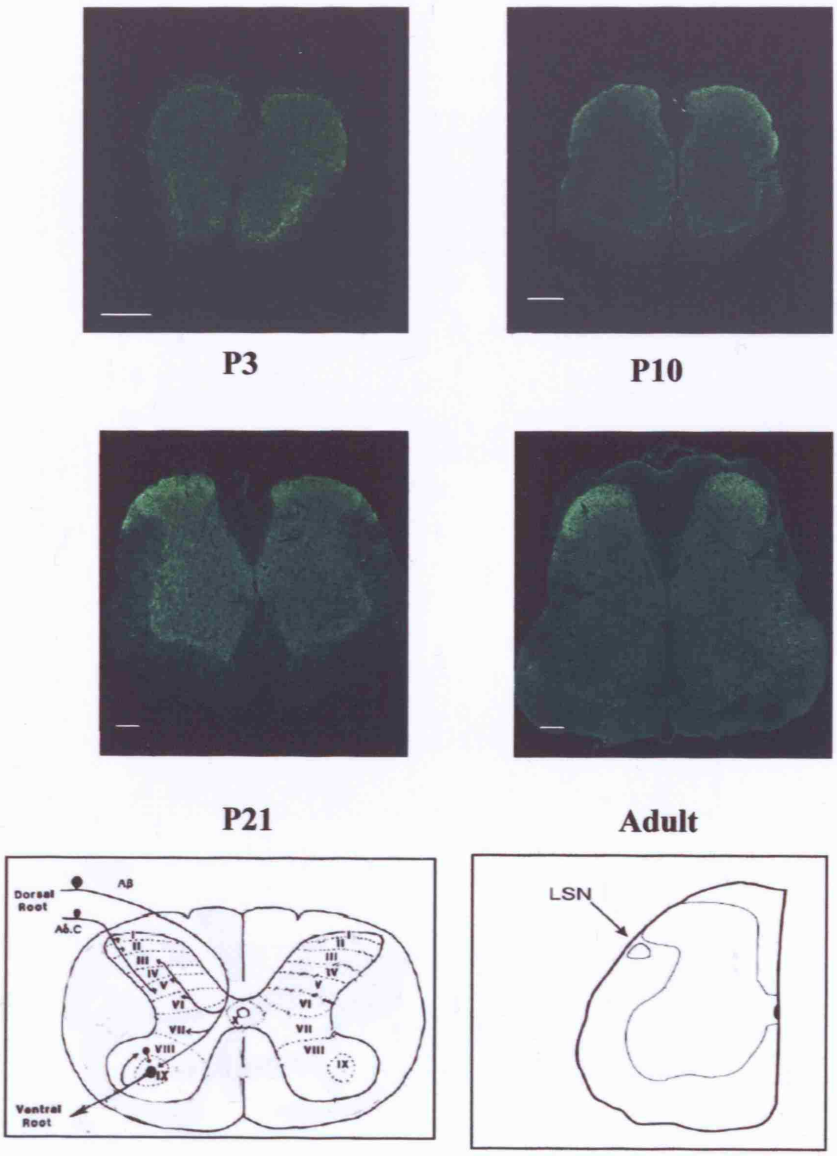


Fig 2.9

Developmental expression of KCC2 in the spinal cord of rats. At P3 KCC2 is seen in the ventral horns, by P10, some can also be seen dorsally. A 'band' of expression in the superficial dorsal laminae begins around P21, and is clearly seen in the adult tissue. Scale bars 200 μm. A laminar map of the lumbar cord is shown for orientation.

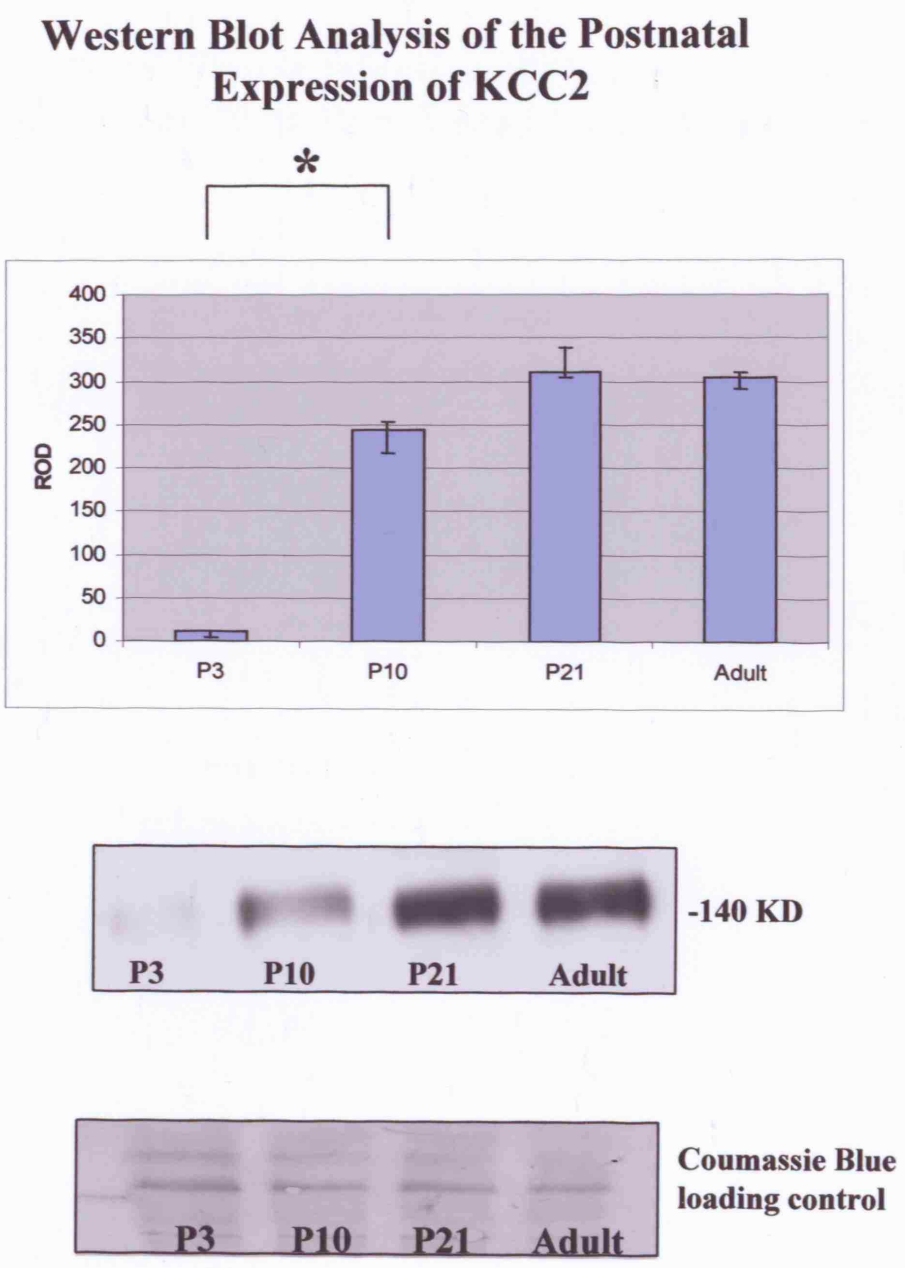
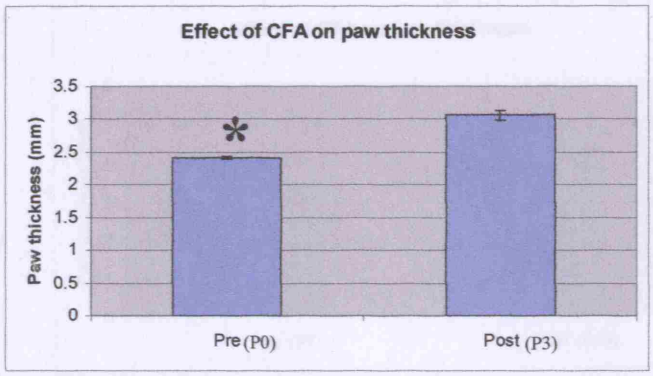


Fig 2.10
Western blot analysis demonstrating the post-natal up-regulation of KCC2. n=6 at all ages. * $P<0.05$ (ANOVA)

Effects of Plantar Injection of Complete Freud's Adjuvant at P0 on Rats Tested 3 Days Later at P3



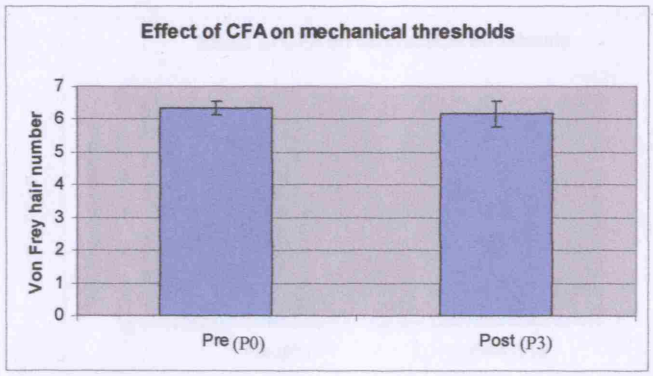
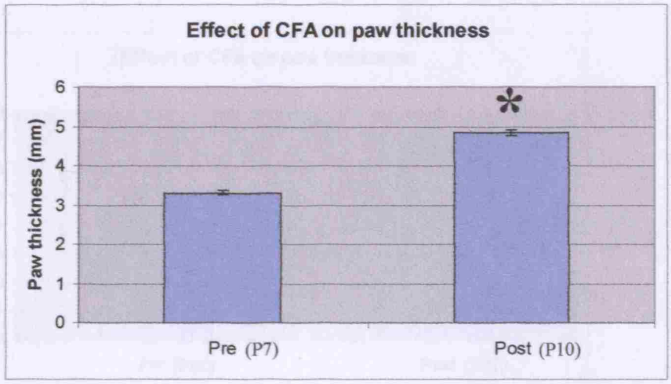


Fig 2.11

Comparison of paw thickness and mechanical thresholds before and three days after CFA injection at P0, n = 6. Paw thickness is significantly increased 3 days after the injection* $P < 0.05$, but mechanical threshold remains unchanged at this age (ANOVA)

Effects of Plantar Injection of Complete Freud's Adjuvant at P7 on Rats Tested 3 Days Later at P10



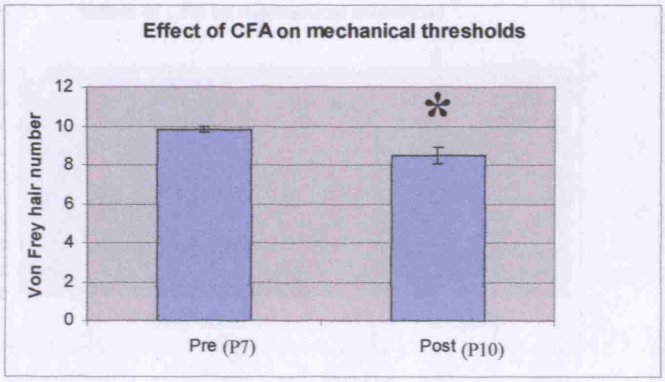


Fig 2.12

Comparison of paw thickness and mechanical threshold in P7 rats before and 3 days after intra-plantar injection of CFA . n = 6. Both paw thickness an mechanical threshold are significantly altered. * $P < 0.05$ (ANOVA)

Effect of Plantar Injection of Complete Freud's Adjuvant at P18 on Rats Tested 3 Days Later at P21

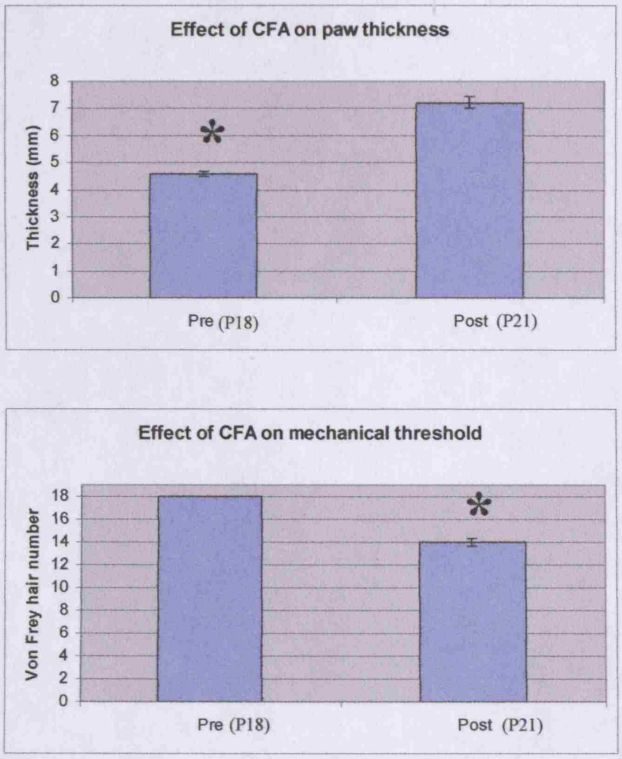
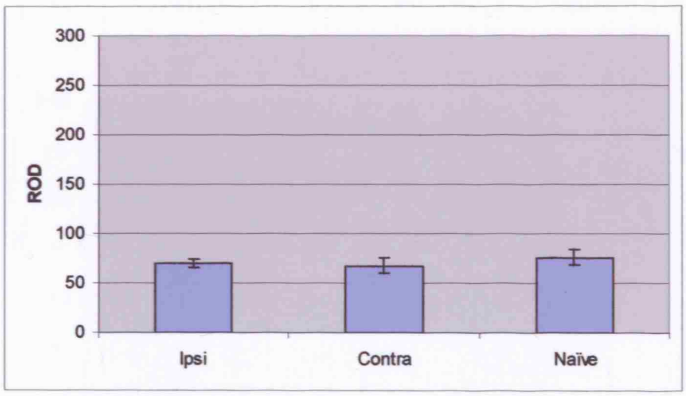
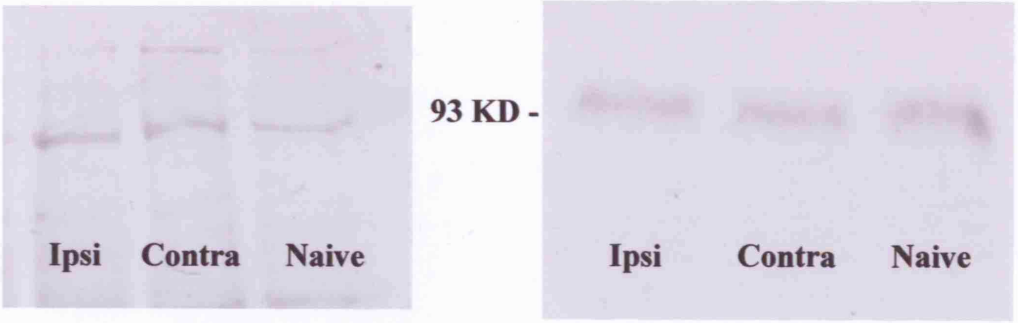


Fig 2.13

Comparison of paw thickness and mechanical threshold at P18 before and 3 days after intra-plantar injection of CFA. n = 6. Both paw thickness and mechanical thresholds are significantly altered.* $P < 0.05$ (ANOVA).

Expression of Gephyrin in P3 Rat Pups Following Plantar Injection of CFA at P0



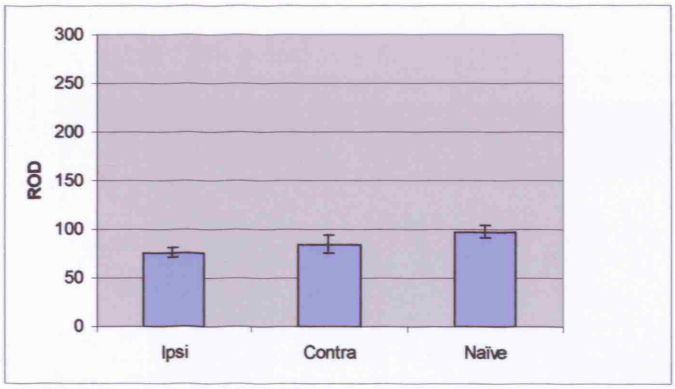


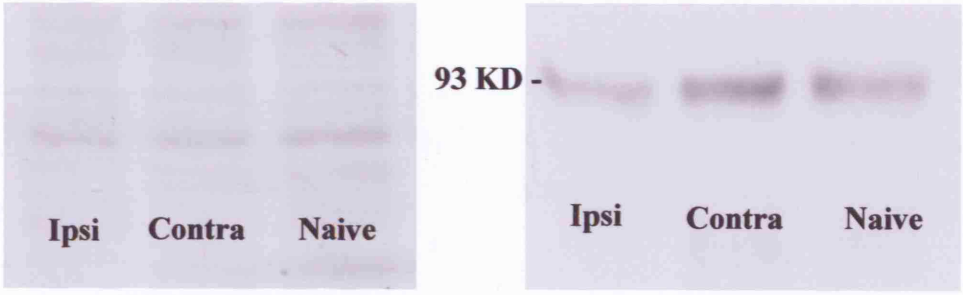
Coumassie Blue loading control **n=6 for all conditions**

Figure 2.14

Western Blot analysis comparing Gephyrin expression in the ipsilateral and contralateral dorsal horns of P3 rat pups with that of naïve animals of the same age (ROD = relative optical density). Gephyrin expression at P3 does not appear to be affected by CFA injection.

Expression of Gephyrin in P10 Rat Pups Following Plantar Injection of CFA at P7

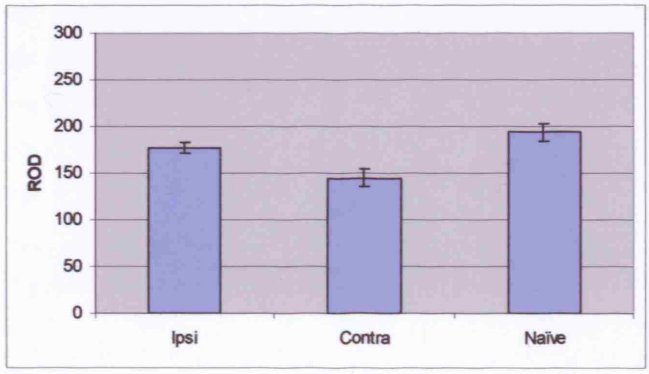


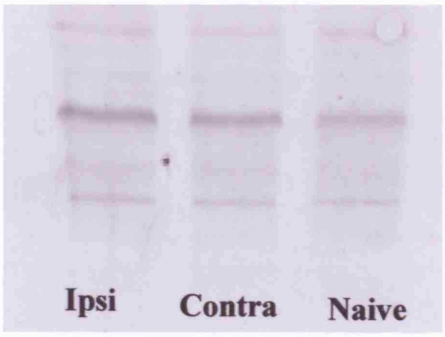


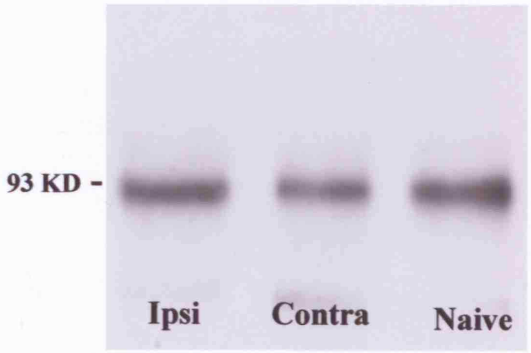
Coumassie Blue loading control **n=6 for all conditions**

Figure 2.15
Western Blot analysis comparing Gephyrin expression in the ipsilateral and contralateral dorsal horns of P10 rats with that of naïve animals of the same age (ROD = relative optical density). No significant difference is demonstrated.

Expression of Gephyrin in P21 Rats Following Plantar Injection of CFA at P18





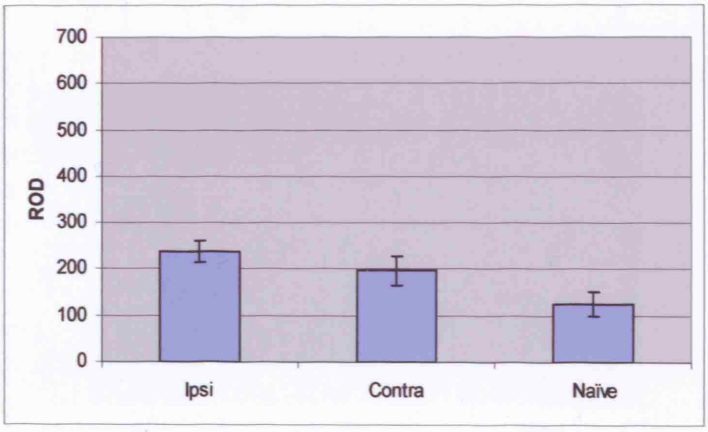


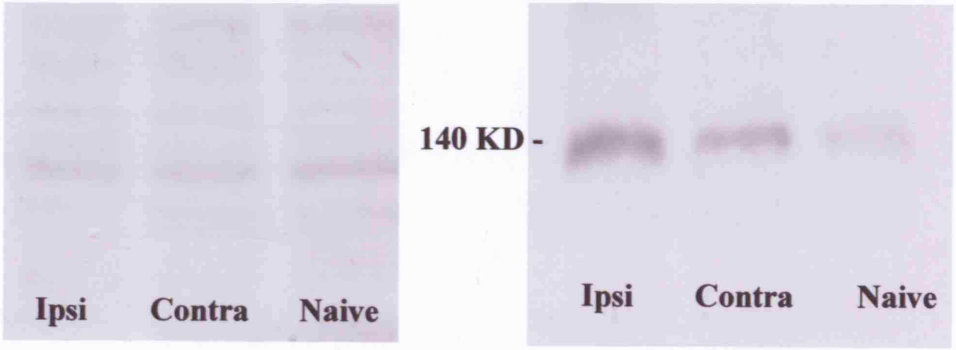
Coumassie Blue loading control

n=6 for all conditions

Figure 2.16
Western blot analysis comparing Gephyrin expression in the ipsilateral and contralateral dorsal horn of P21 rats with that of naïve animals of the same age (ROD = relative optical density). Again CFA does not appear to significantly affect the expression of gephyrin.

Expression of KCC2 in P3 Rats Following Plantar Injection of CFA at P0





Coumassie Blue loading control

n=6 for all conditions

Figure 2.17
Western Blot analysis comparing KCC2 expression in the ipsilateral and contralateral dorsal horn of P3 rat pups with that of naïve animals of the same age (ROD = relative optical density). No significant different was found at this age.

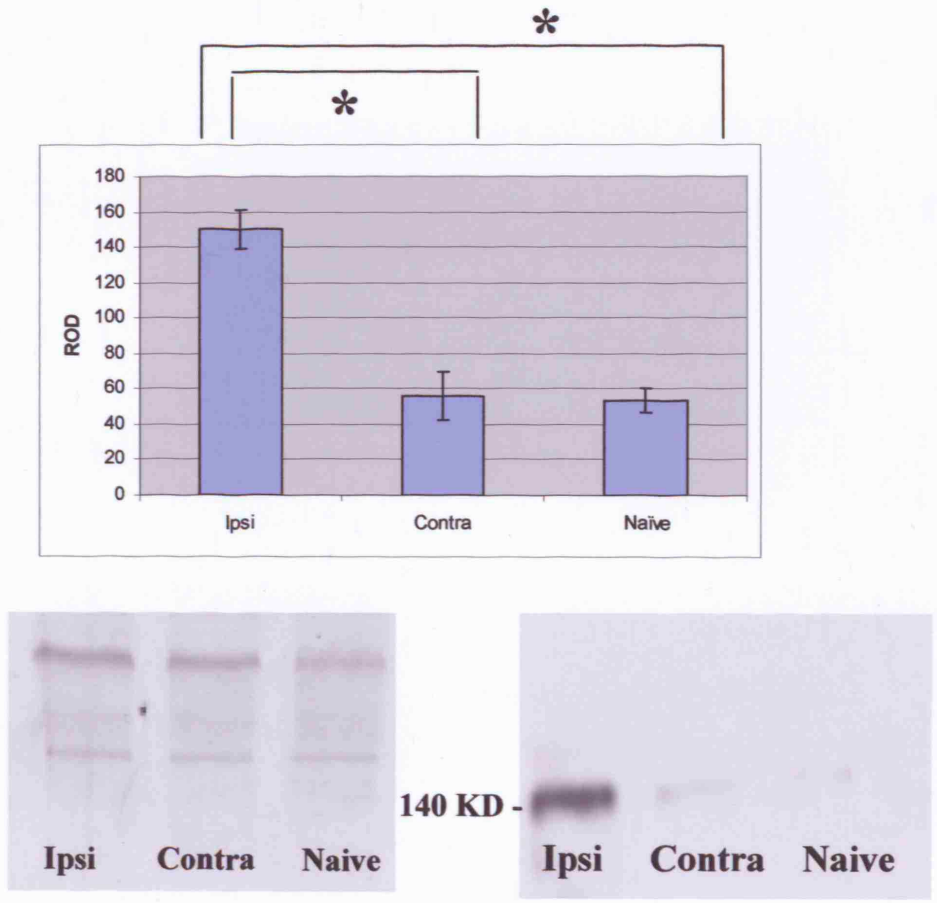
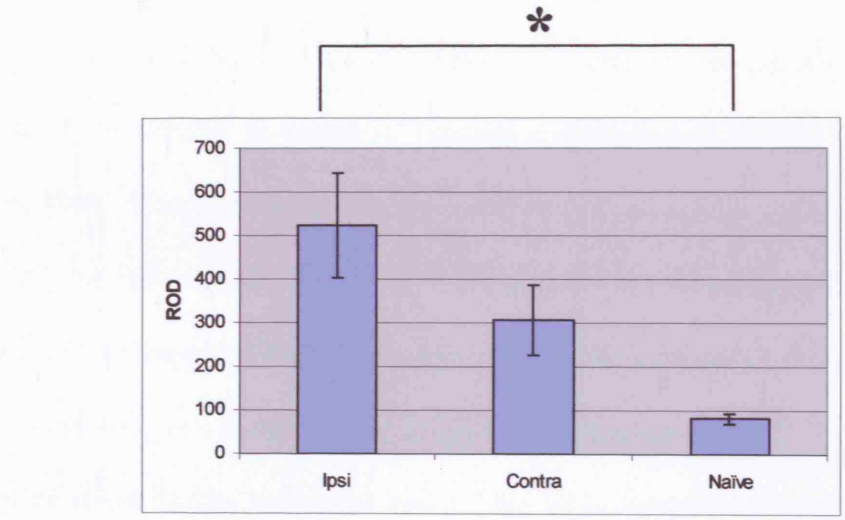
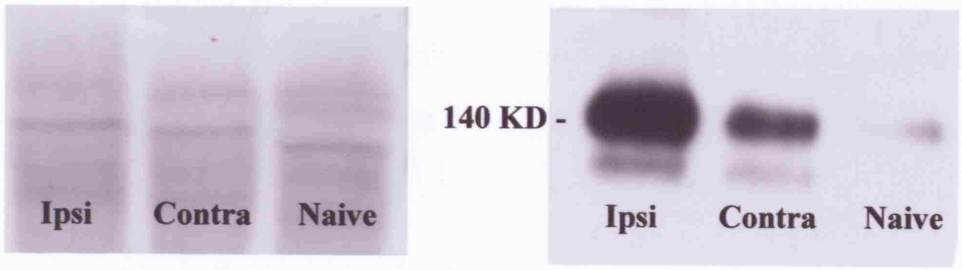


Figure 2.18

Western Blot analysis comparing KCC2 expression in the ipsilateral and contralateral dorsal horns of P10 rats with that of naïve animals of the same age. The expression in the ipsilateral dorsal horn is found to be significantly higher than both contralateral and naïve ($P<0.05$, ANOVA).

Expression of KCC2 in P21 Rats Following Plantar Injection of CFA at P18





Coumassie Blue loading control **n=6 for all conditions**

Figure 2.19
Western Blot analysis comparing KCC2 expression in the ipsilateral and contralateral dorsal horns of P21 rats with that of naïve animals of the same age. The KCC2 expression on the ipsilateral side is significantly higher than in naïve animals ($p<0.05$, ANOVA).

2.4 Discussion

2.4.1 Anatomical changes occurring in the postnatal dorsal horn

The spinal cord develops ventrodorsally, with the neurons of the substantia gelatinosa being the last to form. Within this region, the relatively sparse projection neurons develop ahead of the numerous local interneurons (Bicknell & Beal, 1984). Although immature, a degree of laminar architecture is visible in the dorsal horn from birth. This is reflected in the relative differences in the density of neuronal nuclei seen here at different levels of the dorsal horn, when the tissue is stained with NeuN. The compact presence of nerve cell nuclei in Lamina II outer stands out from the lesser cell density of lamina II inner, with lamina I consisting of a dorsal-most strip of more widely separated cells. This is in keeping with the observations of Coimbra et al, who studied the postnatal development of lamination in rat cord using Toluidine blue (Coimbra et al, 1986). The ratio of grey matter: white matter is also shown to change postnatally, in NeuN stained sections, as myelinated fibre tracts develop.

The arrival of C fibres in to the dorsal horn has been implicated in the initiation of a number of developmental maturational processes. Most of the C fibres present at birth are known to be of the peptidergic (CGRP+ve) type, with IB4+ve fibres maturing postnatally (Bennett, 1996). This IB4 expressing subset of fibre synaptic terminals cannot be detected in the dorsal horn until P5, despite being present in dorsal root ganglia from E18, suggesting that they form central connections later than peptidergic fibres (Fitzgerald, 2005). Correspondingly, in the immunohistochemical stains performed, IB4 immunoreactivity was not detectable at P3 in the dorsal horn, but began to form a band in lamina II inner at P10, which grew in intensity

thereafter, consistent with the known adult pattern of expression (Hwang et al, 2001). CGRP was present throughout, however, at P3 the band of expression was thinner and appeared relatively limited to laminae I & II outer, before expanding through lamina II by P10, resulting in the previously reported wide band extending throughout the superficial dorsal horn, being most strongly expressed laterally in lamina I (McNeill et al, 1988).

2.4.2 The postnatal developmental expression of gephyrin

In keeping with its role as a synaptic anchoring protein at inhibitory synapses, gephyrin staining was punctate and principally limited to cell membranes. Gephyrin expression appears to move superficially up the dorsal horn in a ventrodorsal manner, analogous to the developmental progression of GABA and glycine at an earlier stage of development (Allain et al, 2004; Watanabe et al, 1995). This developmental sequence was confirmed by co-localisation studies with CGRP, known to demarcate the outer rim of the dorsal horn (laminae I & II). At P10, there was a clear gap between the gephyrin staining in the lower dorsal horn and the CGRP immunofluorescence, whereas in the adult there was a definite overlap between the two. As the band of gephyrin staining moves dorsally, and increases in intensity (around P21), a discrete area of diffuse, punctate staining becomes apparent in lamina I. This is likely to represent a population of giant neurons in this region known as Waldeyer cells. This group of cells has previously been shown to be 'gephyrin-rich' and unlike many lamina I cells to be mostly NK1-ve. They have been demonstrated to project to the parabrachial area by the use of retrograde labelling (Puskar et al, 2001), and as such are likely to play a role in pain processing. It follows that in the immediate postnatal period, gephyrin is found ventral to the

position of the inhibitory interneurons and as such offers them no post-synaptic scaffolding support, it is possible that a developmental signal, such as the arrival of C fibres in the dorsal horn, is needed to trigger the maturation of the protein's distribution.

In this study, quantitative expression of gephyrin has been shown by western blot analysis to be significantly lower at P3 and P10 than in mature dorsal horn. This is likely to have functional implications for sensory processing in the immature dorsal horn. Depletion of gephyrin; either using antisense oligonucleotides in cultured spinal neurons (Kirsch et al, 1993), or gene targeting in mice (Feng et al, 1998); has been shown to significantly reduce glycine receptor clustering. Behaviourally, gephyrin-null mice have been shown to become increasingly sensitive to tactile stimuli, before eventually succumbing to respiratory failure (Feng 1998). Despite the presence of functional glycine receptors, patch clamp data from superficial dorsal horn neurons has also shown a relative absence of miniature inhibitory post-synaptic currents before the age of P10 (Baccei & Fitzgerald, 2004). It is therefore conceivable that this lack of synaptic clustering of inhibitory receptors could greatly reduce the effectiveness of inhibitory interneurons, as synapses with large gephyrin-rich post-synaptic receptor areas are likely to be of greater synaptic strength. This could manifest in the dorsal horn itself, by limiting segmental inhibition of noxious input, as well as in brainstem areas such as the rostroventral medulla (RVM), important in processing descending inhibition. It is also possible that the Waldeyer cells projecting to the parabrachial area, mentioned above, may be relieved of their inhibitory control. These mechanisms could all contribute to the relative lack of inhibition known to occur in the immature nervous system.

2.4.3 Factors controlling the postnatal upregulation of gephyrin

The factors controlling the developmental upregulation of gephyrin remain to be fully elucidated. Blockade of action potentials by the use of high dose tetanus neurotoxin in adult cat abducens neurons has been shown to reduce the number of gephyrin immunoreactive clusters in the soma of abducens motoneurons by 60% (Gonzalez-Ferrero et al, 2004), thus suggesting that the maintenance of gephyrin clusters may be activity dependent. Studies of axotomised facial nerve nuclei in rats have also shown a reduction in gephyrin clusters (Eleore et al, 2005). However, interestingly, in this later study, tetrodotoxin, cardiotoxin and botulinum toxin did not produce a significant effect, leading the authors to conclude that gephyrin cluster density was dependent on intact neuronal connections but not on synaptic activity per se. Similarly, another group has shown that unilateral electrolytic lesions of the vestibular complex of the goldfish, causing degeneration of glycinergic afferent of the Mauthner cell, lead to a reduction in gephyrin-glycine receptor clusters, which was not reproduced by strychnine treatment (Seitanidou et al, 1992). They suggested that glycine-gephyrin complex might be regulated by 'trophic' factors rather than by transmitter-evoked synaptic activity. Models of inflammation provide a method of studying increased sensory activity in the absence of nerve damage, as exaggerated sensation of pain is a cardinal symptom of inflammation. In the study described in this chapter, complete Freund's adjuvant (CFA) was administered to the plantar surface of the hindpaw of rats aged P3, P10 and P21, and the levels of gephyrin were compared on both the ipsi- and contra-lateral sides with those of a naïve animal of the same age. The paw thickness was measured 3 days after injection and significant swelling was found at all three ages. In accordance with previous reports, the already

low mechanical withdrawal thresholds of P3 pups were not significantly reduced after CFA injection, but the older animals were significantly more tender (Walker et al, 2003). No difference was found between the inflamed animals and the naïve controls at any of the three ages. This suggests that increased noxious sensory input does not accelerate the postnatal upregulation of gephyrin. If the developmental signal for the upregulation of gephyrin was simply an increase in afferent input, an inflammatory insult might have been expected to provide an accelerated drive. As no difference was observed between the inflamed and naïve adult animals, the effect of inflammatory injury also appears to be different to that previously reported for nerve injury models. If, as previously suggested, the maintenance of gephyrin clusters is dependant on intact neuronal input, but not synaptic activity, then the results presented here may be considered to be in agreement, suggesting that neither an increase nor a decrease in synaptic input has a significant effect. The signal driving maturation may be more specific, for instance involving the arrival of C fibres in to the dorsal horn; this could be investigated by repeating the experiment in capsaicin treated pups.

2.4.4 The postnatal developmental expression of KCC2

On the cellular level, expression of KCC2 was principally limited to cell membranes, in keeping with its trans-membrane transporter role. Some KCC2 was visible in the grey matter from P3, but this was limited to the ventral horn, and appeared to be located on the large cell bodies of developing motoneurons. From P10 onwards a dense band of expression begins to form in the superficial dorsal horn, increasing in width and intensity with advancing age. Thus, KCC2 appears to develop in a ventrodorsal fashion, analogous to that of GABA and glycine described previously

(Allain et al, 2004; Watanabe et al, 1995). Between the ages of P10 and P21, some fibres extending medially between the dorsal horns were brightly stained for KCC2. This phenomenon appears to be transient, as it is not seen in adult sections. Interestingly, Allain et al noted GABA immunoreactive fibres in the dorsal white matter during development, which were seen to decline in numbers around the time of birth. It may be possible that the transient expression of KCC2 seen here is related to the same fibres. A discreet, lateral area of KCC2 immunoreactivity can be seen to appear from around P21 in an area corresponding to the lateral spinal nucleus (LSN). Retrograde tracer studies have shown LSN neurons to project to the periaquaductal grey matter, thalamus and nucleus accumbens (Cliffer et al, 1991); and to receive descending projection from the raphe nucleus, brainstem reticular formation dorsal column nuclei and periaqueductal grey matter (Jiang et al, 1999). Peripheral noxious thermal stimulation has been shown to increase C-fos expression in the area (Olave et al, 2004) strongly suggesting a role in the processing of noxious input. It has been shown that synaptic activities of LSN neurons are less responsive to natural stimulation than electrical stimulation of the dorsal root, the role of the LSN in sensory processing may therefore be particularly important in hyperalgesia (Jiang, 1999). Thus, KCC2 expression in this area is likely to be expressed on inhibitory interneurons modulating nociceptive processing.

KCC2 has been demonstrated to be up regulated in the first weeks of life in many areas of the CNS including the hippocampus (Ludwig et al, 2003); retina (Leitch et al, 2005); cerebellum (Stein et al, 2004); and auditory brainstem (Balakrishnan et al, 2003). A recent study by Stein and co-workers suggested that KCC2 levels in the spinal cord do not change beyond the age of P3 (Stein et al, 2004), reaching a plateau

much earlier than elsewhere in the CNS. However, Stein's study failed to differentiate between dorsal and ventral cord, and it has long been recognised that the motoneurons are indeed some of the first to mature, with KCC2 mRNA detectable from 12.5 (Li et al, 2002). KCC2 knockout mice die almost immediately after birth due to mechanical respiratory failure (Hubner et al, 2001), thus revealing an essential role for KCC2 in the motor system from birth onwards. Correspondingly, the pattern of immunofluorescence seen in the work presented here shows early KCC2 staining in the ventral horns, before any detectable expression in the dorsal horn.

In the current study, the ventral and dorsal horns were carefully separated by longitudinal sectioning of the lumbar spinal cord, allowing quantification, by western blot analysis, of KCC2 in sensory neurons without contamination from the documented high levels in the motoneurons. This analysis of isolated dorsal horn tissue revealed a significant up regulation of KCC2 between P3 and P10, with a further small increase between P10 and P21. Levels in adult tissue were similar to those at P21. Low levels of KCC2 in the early development of the dorsal horn could have a significant functional impact. The output of GABA and glycinergic interneurons has the potential to be qualitatively different, due to the impact on their electrochemical gradient. Indeed, in many regions of the immature CNS $GABA_A$ receptor activation causes membrane depolarisation that is sufficient to elicit action potentials due relative lack of KCC2 (Ben Ari, 1989). In patch clamp recordings from superficial dorsal horn neurons, Baccei & Fitzgerald showed that between P0 and P2, GABA application produced depolarisation in 40% of neurons, although this failed to reach action potential threshold (Baccei & Fitzgerald, 2004). By one week of age, in the same study, GABA evoked only membrane hyperpolarisation

suggesting that the switch from excitation to inhibition, beginning in utero, is complete by one week after birth. In accordance with this, the largest increase in KCC2 in the data presented in the current study was found to be between P3 and P10, and it therefore appears possible that the threshold level of KCC2 required for mature chloride handling is reached at around this stage. This suggests that a critical period is likely to exist in the first week of life, during which the upregulation of KCC2 occurs, it would seem possible that disruption of this process could have far reaching consequences.

2.4.5 Factors controlling the postnatal upregulation of KCC2

The factors governing the up regulation of KCC2 remain the subject of much debate (see section 1.8.3). There is a lack of agreement as to whether the changes occurring during development are activity dependent and if so what the nature of this activity comprises. Many of the studies conducted to date were performed *in vitro*, using hippocampal neurons in culture (Ganguly et al, 2001; Ludwig et al, 2003). Although this allows free manipulation of neurotransmitter and their antagonists, it removes regulatory influences from other parts of the immature nervous system, which may be important. Here we have used complete Freund's adjuvant to produce an inflammatory plantar lesion *in vivo* in rats aged P3, P10 & P21. This acts as a 'naturally occurring' source of increased input to the superficial dorsal horn. The quantitative expression of KCC2 in the dorsal horn ipsi- and contralateral to the inflammation were then compared with that of a naïve animal using western blot analysis. If afferent input alone were acting as a trigger for the upregulation of KCC2, an accelerated pattern might be expected. No detectable difference was found in the very low levels of expression at P3; however, at P10 the expression of KCC2

was significantly greater on the side of the inflammation compared to the contralateral side or to naïve dorsal horn. This suggests an upregulatory effect of inflammatory injury on the immature levels of KCC2 found at P10. The lack of effect at P3 may reflect immaturity of other contributory parts of the sensory system, or suggest a threshold before which KCC2 in the dorsal horn is itself not 'inducible'. In a similar study, cochlear ablation prior to the onset of hearing was used as an *in vivo* method of reducing input to the lateral superior olive (LSO) of the rat (Shibata et al, 2004). The authors found that intracellular chloride remained high and KCC2 mRNA low in the animals that had undergone cochlear ablation. They were able to partially reproduce their results by using intra-cochlear strychnine pellets instead of cochlear ablation, suggesting that glycine activity is particularly important. A further *in vivo* study has been carried out using bicuculline pumps implanted in to the eyes of infant turtles. The expression of KCC2 in the retina of the hatchlings, failed to show its usual upregulation at day 28 post-hatch in the presence of the $GABA_A$ antagonist (Leitch et al, 2005). This supports the importance of neuronal activity in the up regulation of KCC2 and suggests a role for GABA signalling. Together these results show that reducing normal sensory input appears to prevent the postnatal upregulation in KCC2, whereas increased activity appears to enhance the developmental increase in the chloride transporter.

2.4.6 Modulation of KCC2 in the mature CNS

In view of the plasticity demonstrated in the postnatal development of chloride transport, it is interesting to note that a number of authors have reported changes in KCC2 levels in mature neurons the face of pathological conditions. Several studies have shown a drop in KCC2 with a corresponding depolarising action of GABA /

glycine after nerve injury (Nabekura et al, 2002; Coull et al, 2003; Toyoda et al, 2003), as well as in other conditions associated with neuronal damage, such as models of epilepsy (Rivera et al, 2002). Interestingly, inflammatory models of pain appear to produce a quite different response. In the adult rat, the present study using intra-plantar CFA mediated inflammation shows a bilateral increase in KCC2 in the dorsal horn compared to naïve animals. This was found to be more pronounced on the ipsilateral side, and did not attain statistical significance contralaterally. A similar study using CFA injection around the tibiotarsal joint of rats as a model of acute arthritis also found an increase in KCC2 and KCC2 mRNA bilaterally in the dorsal horn, with a more pronounced effect on the ipsi-lateral side. Immunohistochemical analysis in this case revealed that the changes in KCC2 were limited to the superficial laminae only (Moralez-Aza et al, 2004). In inflammation, neuronal activity is usually increased without the confounding variable of neuronal injury, and therefore it follows that the cellular mechanisms involved may be different. The increase in KCC2 seen in chronic inflammation may provide a physiological down regulation of the chronic pain, whereas the decrease in KCC2 following nerve damage may be a pathological phenomenon contributing to neuropathic pain.

Contralateral effects, which are qualitatively similar to those occurring on the ipsilateral side, but of a smaller magnitude, have been documented in a number of pain models (Koltzenburg et al, 1999). The aetiology of these is thought to involve a central mechanism, in particular signalling via the system of commissural interneurons that is present in the spinal cord and brainstem. The absence of this phenomenon at P10 where only an ipsilateral effect is found could therefore be a reflection of immaturity of these commissural interneurons.

2.4.7 Conclusion

In this chapter, known neuronal stains (NeuN, CGRP, and IB4 antibodies) have been used to demonstrate the changes in neuronal density and grey: white matter ratio occurring postnatally, as well as the postnatal maturation of C fibre terminals in the dorsal horn. Gephyrin expression has been shown to be significantly lower in rat pups under the age of P10, with a possible quantitative impact on inhibitory processing. The previous suggestion that the maintenance of gephyrin clusters is dependent on intact neuronal connections, but not directly on synaptic activity is supported by the lack of any effect of inflammatory injury on the up-regulation of the protein. In the case of postnatal development, the late maturing C fibres of the dorsal horn are an attractive candidate as a regulatory signal for gephyrin, as a lower percentage of dorsal horn neurons with nociceptive inputs are observed in the first week of life compared to in adults (Jennings & Fitzgerald, 1998). Separation of the dorsal and ventral parts of the lumbar spinal cord has allowed us to focus upon the dorsal horn and show a marked upregulation of KCC2 in the neonatal lumbar dorsal horn between P3 and P10. This change is shown to be activity dependent around the age of P10, by the use of a model of acute inflammatory pain. Acute plantar inflammation in the adult animal produces a bilateral increase in KCC2 in the dorsal horn, consistent with other models of inflammation described in the literature, but qualitatively different to the effect of nerve injury models. This highlights the differences between commonly used models of pain.

Chapter Three

In vitro culture of isolated neonatal dorsal horn neurones

3.1 Introduction

The normal development of synaptic connections in the central nervous system is a complex process that ultimately determines the functional integrity of adult neuronal processing. A complex developmental refinement of connections takes place both before and after birth in the mammalian spinal cord, involving both activity dependent and activity independent mechanisms (Ren et al, 2004; Waldenstrom et al, 2003; Andrews & Fitzgerald, 1999; Beggs et al, 2002). Most of our understanding of activity dependent maturation comes from the study of excitatory synaptic transmission in regions such as the visual system (Stryker & Harris, 1986) or the neuromuscular junction (Colin-Le Brun et al, 2004) which are known to require normal patterns of spike activity for appropriate connections to be formed. However, synaptic inhibition can also be regulated by synaptic activity. There is an ongoing debate regarding the activity dependence of the postnatal upregulation of the neuron specific cation chloride co-transporter KCC2, which is causally linked to the switch from excitation to inhibition during development (Rivera et al, 1999). It is widely agreed that the transporter is developmentally regulated in several areas of the CNS, including the hippocampus (Ludwig et al, 2003), retina (Sernagor et al, 2003), and cerebral cortex (Zhu et al, 2005). Nevertheless, the molecular mechanisms responsible for triggering and thereafter progressively upregulating KCC2 expression during development have remained elusive. Several authors disagree on whether GABA and/or glutamatergic activity is needed for the maturation of KCC2 expression, with *in vitro* studies of hippocampal neurons reporting quite different results (Ganguly et al, 2001; Ludwig et al, 2003).

In chapter 2, evidence was presented that the postnatal development of KCC2 was regulated by peripheral sensory activity by the use of an *in vivo* model of inflammation. Peripheral injury caused an accelerated upregulation of KCC2 and it was proposed that this was due to increased afferent activity produced by the injury. In order to confirm this observation, direct manipulation of the conditions surrounding the dorsal horn neurons during this critical phase of their development was needed, and therefore an *in vitro* culture model was used. The generation of primary cultures of neuronal cells allows the examination of their functional maturation in the absence of glial cells and in a defined molecular environment. Thus, an existing protocol for the culture of neurons dissociated from the most superficial laminae of the neonatal rat spinal cord dorsal horn (Jo et al, 1998) was adapted with the kind assistance of Dr Sarah Slack. In this chapter, the method is validated by studying the morphology of growing neurons, and by examining their immunohistochemical properties. Cells are counted and their survival *in vitro* is measured. The upregulation of KCC2 in the dissociated cultured neurons is then quantified in terms of numbers of cells expressing the protein and compared with *in vivo* data. Finally, the culture medium is manipulated by the addition of:

- The sodium channel blocker tetrodotoxin (TTX), in order to block action potentials;
- Supra-physiological concentrations of potassium, in order to increase cellular excitation; and
- Receptor inhibitors (GABA, glycine, glutamate)

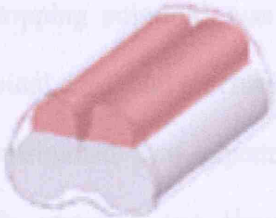
The effect of the agents above on the expression of KCC2 is measured by comparison with levels of staining for MAP2, a known neuronal marker.

3.2 Materials and Methods

All reagents used were of the highest-grade purity or tissue culture grade where necessary, and purchased from Sigma-Aldrich (UK), unless otherwise stated. Ultra-pure water (18MΩ) was used for all buffers. One-day-old Sprague Dawley rats of both sexes, bred by the Central Animal Facility, University College London; were used for dorsal horn neuron harvest. The animals were kept in artificial lighting on a 12:12h light cycle and the temperature was kept constant at 21°C. Food and water were given *ad libitum* and all procedures were carried out in accordance with the United Kingdom Animal Procedure Act 1986. Rat pups were housed with their mother and littermates.

3.2.1 Dorsal Horn Neuron Cell Culture

Cultures of dorsal horn neurons were prepared from one-day-old rat pups (see above), according to methods adapted form Schlichter and colleagues (Jo et al, 1998a; Jo et al, 1998b; Jo & Schlichter, 1999; Hugel & Schlichter, 2000). The instruments and preparation were kept sterile at all times by means of heat sterilisation and/or alcohol wash. The solutions were sterilised by filtration (0.4μM diameter pores), and procedures were conducted under a Class 2 laminar flow hood. Rats were placed on ice and rapidly decapitated. The dorsal skin was removed and a laminectomy was performed in a caudo-rostral direction in order to expose the spinal cord. The dura was then carefully removed to avoid trauma to the cord. The dorsal third of the spinal cord was removed with a mounted, flexible razor blade and placed in a 15 ml falcon tube of ice-cold sucrose buffer (sucrose 260mM, KCl 2 mM, $MgCl_2$ 0.9 mM, KH_2PO_4 1.2mM, glucose 1mM, $NaHCO_3$ 2.6 mM), oxygenated with 95% O_2 & 5% CO_2.



Portion of cord removed for culture

The dissection procedure was carried out as rapidly as possible in order to maximise cell viability. A maximum of 10 animals (or one litter where littermates numbered less than 10) were used at any one time, so that an average of 30 minutes was taken in total for the collection of tissue. The dorsal cord fragments were then transferred to a further 15ml falcon tube containing pre-warmed (37°C) enzymatic digestion solution (20u/ml, equivalent to 1mg/ml papain), which was made up fresh in a buffer solution of EBSS (Earl's buffer salt solution (GIBCO, UK)) without calcium or magnesium, and oxygenated with 95% O_2 & 5% CO_2. The falcon tube was then placed in a water bath maintained at 36°C, and gently bubbled with 95% O_2 & 5% CO_2.

Following removal of the papain solution, the enzymatic reaction was stopped by adding 1ml of 'stopping solution' (1ml EBSS (with calcium and magnesium, GIBCO, UK) containing bovine serum albumin (BSA) 1mg/ml, trypsin inhibitor 10mg/ml and DNAase 0.01%). The cells were mechanically dissociated by tituration with a trimmed 1 ml tip on a Gilson pipette, 7 times very gently to dissociate the most delicate cells first. The solution was left to stand for 1 minute in order to allow the fragments of tissue to settle to the bottom of the tube. The liquid layer was then removed and gently placed on to a 4 ml aliquot of pre-warmed 'cushion solution' (identical to the 'enzymatic stopping solution' except that the concentration of BSA was 10 fold greater in order to produce a concentration gradient for separating cellular debris from intact cells by centrifugation).

A further 1ml of 'stopping solution' was added to the falcon tube containing the fragments of dorsal spinal cord, and mechanical dissociation was performed once again, this time with an untrimmed 1ml pipette tip. After being allowed to stand, the supernatant was again removed and slowly added to the 'cushion solution'. The dissociation step was performed a total of 4 times.

The Falcon tube containing 4ml of 'cushion solution' supporting the 4 ml suspension of cells in 'stopping solution' (clearly visible as discrete layers at this stage) was centrifuged for 5 minutes at 500 rpm. The supernatant was then gently removed and discarded. The cells were re-suspended in 2ml of warmed culture medium consisting of MEMα (Life Technologies), to which the following ingredients were added:

- fetal calf serum (Life Technologies) 5%vol/vol;
- heat-inactivated horse serum (Life Technologies) 5% vol/vol;
- penicillin, streptomycin & fungizone solution 50 IU/ml;
- transferrin 10mg/ml;
- insulin 5mg/ml;
- putrescine 100nM; and
- progesterone 20 nM.

The cells were seeded on to 20mm diameter sterile glass cover slips coated in collagen prepared from rat tails (see below), placed in 50mm diameter plastic tissue culture dishes. In the absence of a mechanical cell counter, various volumes of the cell

suspension were added to cover slips, and the results assessed. An optimal seeding density was obtained by adding 165µl of the concentrated cell solution to each cover slip. The cells were left for one hour in the concentrated cell suspension in order to attach to the collagen, after this 2ml of culture medium (see above) were added. Cultures were maintained in an incubator at 37°C in a water-saturated atmosphere (95% O_2 & 5% CO_2). The cells were 'fed' by replacement of half the medium every 3-4 days.

In later experiments, the cell culture medium was altered by the addition of (tetrodotoxin) TTX 5nM, potassium 10 mM, or a receptor inhibitor 'cocktail' consisting of antagonists of GABA, glycine, and glutamate receptors (D-APV 20µM, NBQX 10 µM, Gabazine 10 µM, and strychnine 0.5 µM). In the case of the potassium, the medium was then changed every other day, in order to keep the concentration of potassium elevated.

3.2.2 Preparation of Collagen from Rat-tails

Rat-tails, which had previously been harvested and frozen, were placed in 95% ethanol, in order to sterilise the outer surface. Tendons from 4-5 tails were removed by successively fracturing small pieces of tail-bone (starting from the distal end), and pulling tendons away from the whole tail using sterile bone-cutters. Approximately 5g of tendons were collected in to a sterile dish and washed in distilled water. The tendons were dissolved overnight at 6°C, in a conical flask containing 500ml 0.5M acetic acid with a magnetic stirring bar rotating slowly. The solution was centrifuged for 60 minutes at 20,000g, in sterilised tubes. The supernatant was then transferred to a 20 cm length of

clean 2.5 cm width dialysis tubing. The dialysis tubing was suspended in a 2l conical flask containing one-tenth strength Basal Medium Eagle (BME) at Ph 7.4 and dialysed overnight at 6°C, by the use of a magnetic stirring bar. The resulting collagen is harvested in aliquots of 10 ml into sterile falcon tubes and stored at 6°C for up to 6 months.

3.2.3 Immunohistochemical Studies of Cultured Neurons

Cells were cultured for up to 14 days *in vitro*. They were then fixed in 4% paraformaldehyde for 20 minutes at room temperature, before being washed in phosphate buffered saline (PBS), and blocked in a solution of 0.075% saponin and 5% normal goat serum for one hour. Following this they were incubated overnight at 6°C with primary antibody: mouse monoclonal anti-MAP2 (Chemicon) 1:500; Rabbit polyclonal anti-KCC2 (Chemicon) 1:200; Mouse monoclonal anti-Gephyrin 1:100 (Alexis); diluted in PBS with 0.075% saponin. The following morning, the cells are washed 3 times in PBS for 10 minutes. In the case of gephyrin and KCC2 antibodies, biotin amplification was required. The cells were therefore incubated for 90 minutes in biotinylated antibody of the appropriate species 1:500, followed three further washes and an incubation in Vectastain solutions A & B, before a final 45 minute period in CY3-anti biotin 1:4000. All other antibodies were directly treated with the secondary fluorescent antibody: FITC 1:200 for 2 hours. Finally, the cover slips were covered in Gel Mount (Sigma), and mounted on to slides.

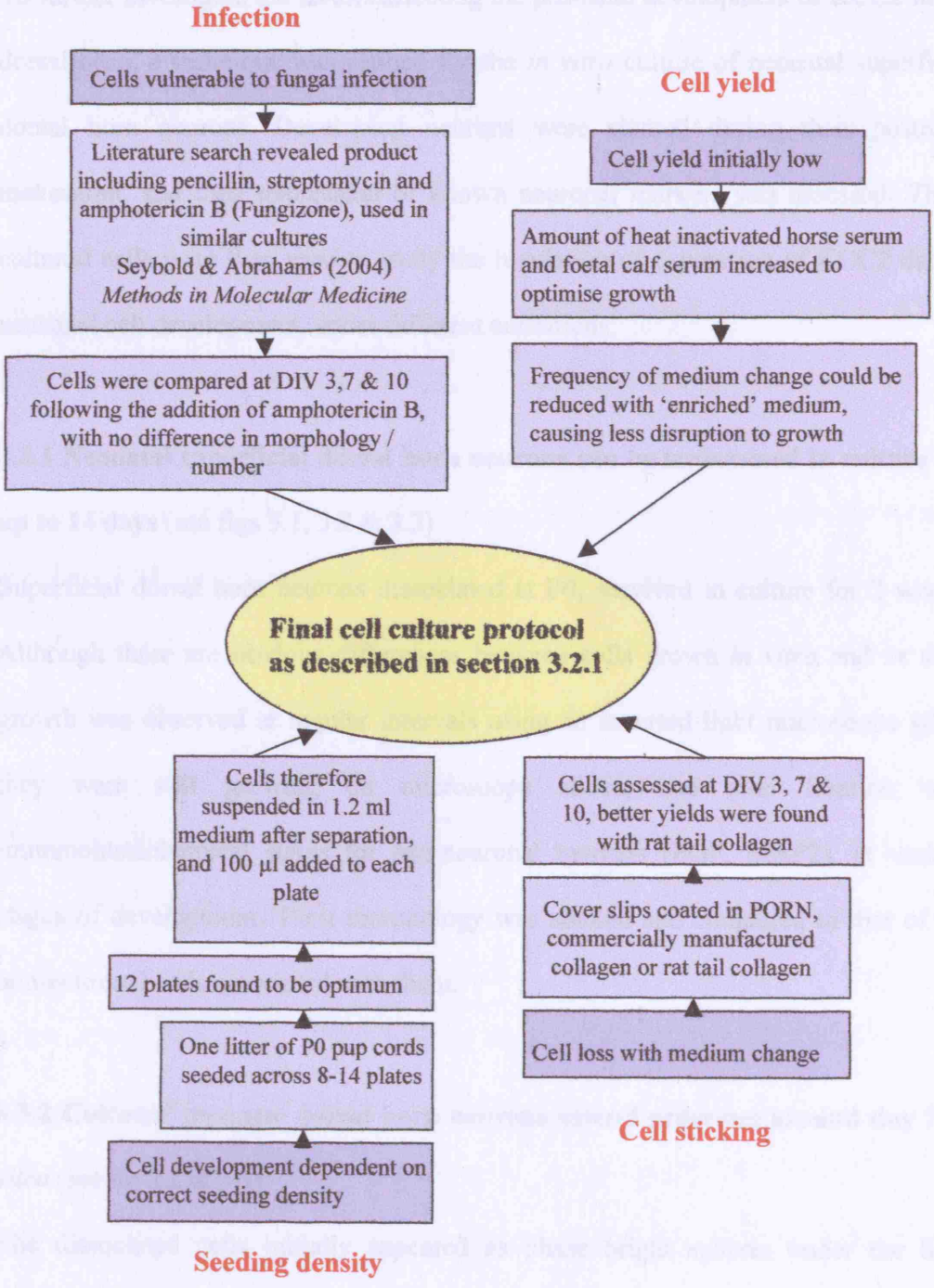
The neuronal markers used to further identify the cells represented in the cultures include:

- IB4 1:200 (Fluorescein Griffonia Simplifolia Lectin I, Vector), & Polyclonal-rabbit-anti-CGRP 1:200 (calcitonin-gene related peptide, Chemicon), which are expressed by superficial dorsal horn neurons;
- Monoclonal mouse anti-NeuN 1:500 (neuronal nuclei, Chemicon), & Polyclonal-rabbit-anti-human PGP9.5 1:500 (protein gene product, Ultraclone), as neuronal markers; and
- Polyclonal-rabbit-anti-reg2 1:1000 (Emergen) a marker of motor neurons, to assess contamination of the sensory neuron culture.

These were all incubated overnight at 6^0C in the concentrations listed before having an appropriate fluorescent secondary antibody applied after careful washing. 12 cover slips were used for each experiment and 3 fields of view were analysed per cover slip with the mean value being used.

3.2.4 Quantification of Cells Expressing Specific Proteins

Cells were visualised using a fluorescent microscope (Eclipse E-800, Nikon with Nikon objectives). Electronic images were captured by a CCD camera (CoolSnapsC5, Roper Scientific Photometric) and MCID imaging software. Cells were counted manually, using high power view fields selected randomly.



Adjustments made to the neonatal dorsal horn neurone culture methods of Schliter and colleges (Jo & Schliter, 1999).

3.3 Results

To further investigate the factors affecting the postnatal development of KCC2 in the dorsal horn, a technique was refined for the *in vitro* culture of neonatal superficial dorsal horn neurons. Developing neurons were studied during their postnatal maturation, and their expression of known neuronal markers was assessed. These cultured cells were then used to study the regulation of expression of KCC2 during neuronal cell development, under different conditions.

3.3.1 Neonatal superficial dorsal horn neurons can be maintained in culture for up to 14 days (see figs 3.1, 3.2 & 3.3)

Superficial dorsal horn neurons dissociated at P0, survived in culture for 2 weeks. Although there are obvious differences between cells grown *in vitro* and *in vivo*, growth was observed at regular intervals using an inverted light microscope while they were still growing on microscope slides; and after fixation with immunohistochemical stains for pan-neuronal markers (PGP, MAP2), at various stages of development. Their morphology was studied and compared to that of the non-neuronal cells associated with them.

3.3.2 Cultured neonatal dorsal horn neurons extend processes around day 7 *in vitro* (see fig 3.2 & 3.3)

The dissociated cells initially appeared as phase bright spheres under the light microscope. They began to enlarge by day 3 *in vitro* (DIV 3), and formed visible processes from around DIV 7. Networks of connected cells were visible at around 10 days.

3.3.3 By day 14, cultured neonatal dorsal horn neurons begin to look unhealthy

(see fig 3.3)

After 2 weeks in culture, the cell processes began to develop a fragmented appearance ('blebbing' of the neurites), and further growth was rarely seen. It appears that at this stage of development, the cells were no longer adequately supported by the medium and began to die. In the quantitative experiments that follow, cells were therefore studied at days 3 and 10 *in vitro.*

3.3.4 The number of surviving neonatal dorsal horn neurons in culture fall over the first 5 days in culture. (see fig 3.4)

Cells were counted at various stages of development (n=12 cover slips, with 3 fields of view counted per cover slip), and numbers were found to decrease in the first 5 days from around 250 neuronal cells per well, to produce a stable population of approximately 75 neurons per well by day 7 *in vitro.* In order to differentiate cells floating off into the medium from actual cell death, the neurons were stained with caspase 3, a specific marker of apoptosis. Expression of caspase 3 was indeed correspondingly high for the first few days before settling to a steady, low level. This suggests that apoptosis is actively occurring during the period of reduction in cell numbers. The high cell loss occurring in these cultures may therefore be a combination of apoptosis in immature neurons and of the failure of some cells to establish themselves *in vitro.*

3.3.5 The background expression of neuron-associated proteins in cultured dorsal horn neurons at day 10 *in vitro* is compatible with in vivo expression in the superficial dorsal horn (see fig 3.5)

MAP 2 was used as a neuronal marker in all later quantitative experiments, as pilot experiments showed it to be more specific for neuronal cells than PGP (used to study the cells' morphology during their development). Inspection of cells revealed that proteins known to be expressed by sub-groups of superficial dorsal horn neurons, such as neurokinin 1 (NK1) and protein kinase C gamma (PKCγ) were relatively abundant in the cultured dorsal horn neurons after 10 days in culture. On the other hand, reg 2 (a protein found principally in motoneurons), and CGRP (a neuropeptide largely restricted to primary afferent neurons and motoneurons) were very poorly represented (n=12 cover slips for each experiment, with 3 fields of view counted per cover slip). This further suggests that the population of cultured neurons consists largely of superficial dorsal horn neurons.

3.3.6 The expression of KCC2 in cultured dorsal horn neurons is developmentally regulated (See fig 3.6 & 3.7)

Neuronal cells in culture were identified by their morphology, and their expression of the pan-neuronal marker MAP2. The number of cells expressing KCC2 was counted at day 3 *in vitro* (DIV 3) and DIV 10; and compared with the total number of neuronal cells. The expression of KCC2 at DIV10 was significantly greater than at DIV3 ($n=12$, $P<0.05$, Student t test). Thus, KCC2 is up regulated in a similar fashion to its expression *in vivo*. This therefore allows further study of the factors influencing the developmental up-regulation, by manipulations of the growth medium contents.

3.3.7 Tetrodotoxin significantly reduces the developmental upregulation of KCC2 in cultured dorsal horn neurons (see fig 3.8 & 3.12)

Tetrodotoxin (TTX) prevents the generation of action potentials by blocking voltage gated sodium channels. This was therefore added to the medium in order to assess the importance of neuronal electrical activity on the upregulation of KCC2. The cell numbers and morphology were not visibly altered by the presence of TTX. Blockade of action potentials from DIV 0 did not affect the level of the minimal expression present at DIV 3 (n=12). However, by DIV 10, cells exposed to TTX with each change of medium, showed a significantly lower expression of KCC2 (n=12, $P<0.05$, student t test). This suggests that activity dependence plays a role in the developmental upregulation of KCC2.

3.3.8 The developmental up-regulation of KCC2 in cultured dorsal horn neurons occurs independently of GABA, glycine and glutamate receptor activity (see figs 3.9, 3.10 & 3.13)

The cell culture medium was further altered by the addition of receptor antagonists to $GABA_A$ (gabazine); glycine (strychnine); and glutamate (D-APV &, NBQX), with the aim of investigating the reliance of KCC2 upregulation on synaptic activity. Again, the number of cells surviving at each stage, and their morphology remained unaltered. The 'receptor inhibitor cocktail' did not produce a significant effect on the upregulation of KCC2, although there was a small, non-significant trend towards a reduction in expression (n=12).

3.3.9 Supra-physiological concentrations of potassium induce a significant acceleration of KCC2 upregulation in cultured dorsal horn neurons (see fig 3.11 & 3.14)

Lastly, the culture medium was altered by the addition of a supra-physiological concentration of potassium, in order to measure the effect of increased excitatory neuronal activity on the upregulation of KCC2. Unfortunately, in this case, the cell numbers were reduced from DIV 3 onwards, and the surviving cells at DIV 10 were in such poor condition that they had to be discarded. Interestingly, by DIV 3 the expression of KCC2 was almost twice the level of the untreated cells, constituting a statistically significant rise ($n=12$, $P<0.05$, Student t test).

Isolated Dorsal Horn Neurones Growing in Culture

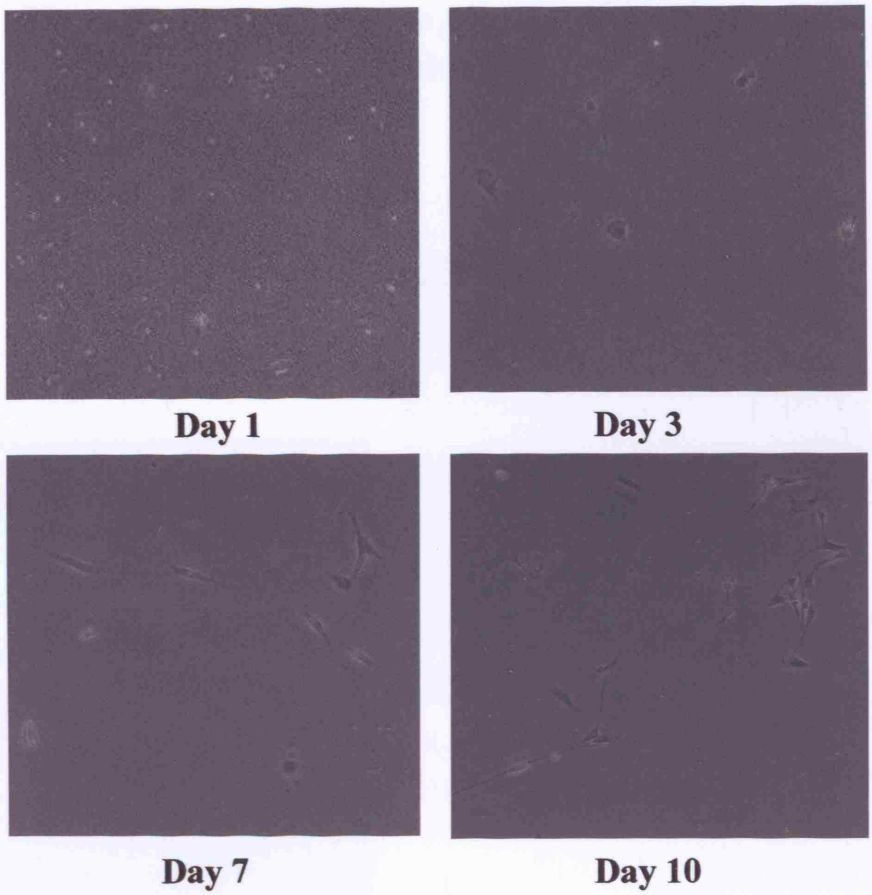
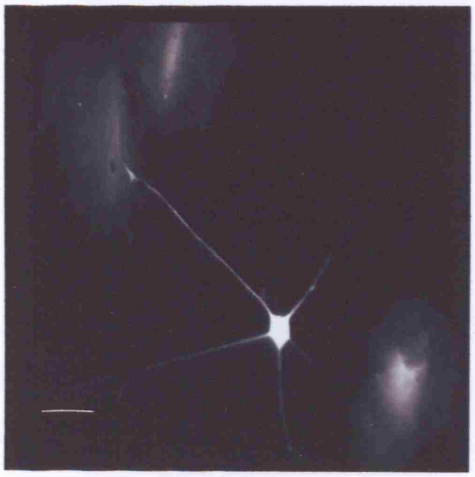


Fig 3.1
Light microscope images (X20) of dorsal horn neurones growing in culture. Neurite outgrowth is shown to be very limited before day 7 in vitro.

Dorsal Horn Neurones Growing Among Non-neuronal Cells in Culture

Central Neuronal cell at day 10 in vitro surrounded by non-neuronal cells



A group of neuronal cells at day 7 in vitro.

Fig 3.2

Examples of the cell populations grown in vitro. Dorsal Horn Neurones can be distinguished morphologically from other cell types by their angular cell bodies and long discreet processes shown above. Scale bar 20μm.

Morphology of Dorsal Horn Neurones Growing in Culture.

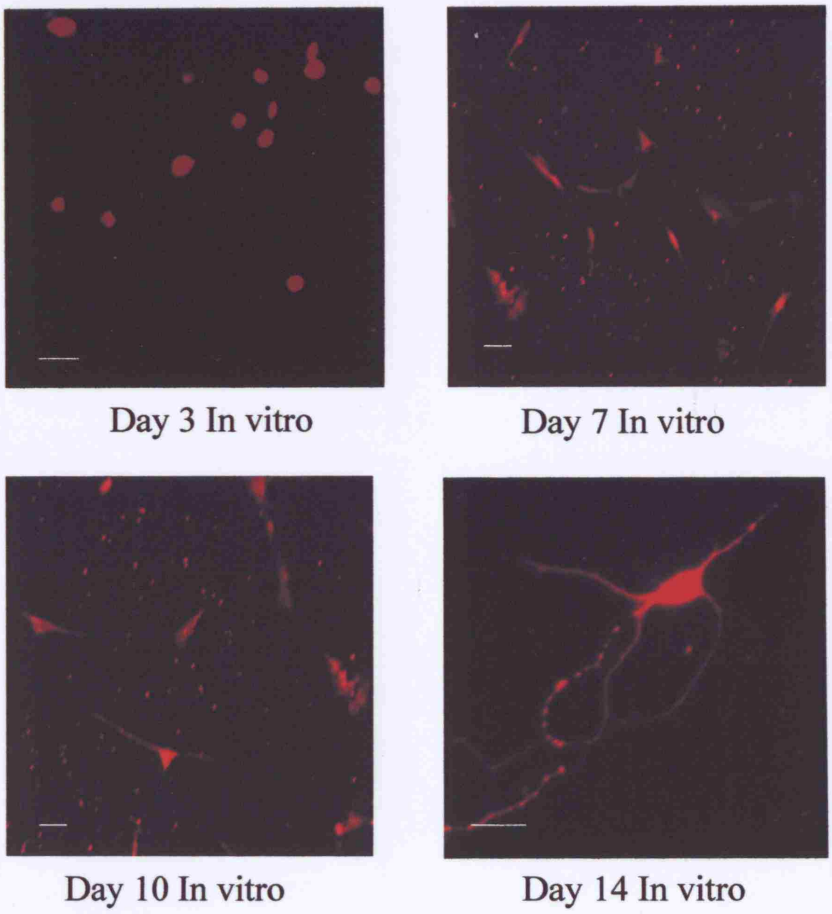
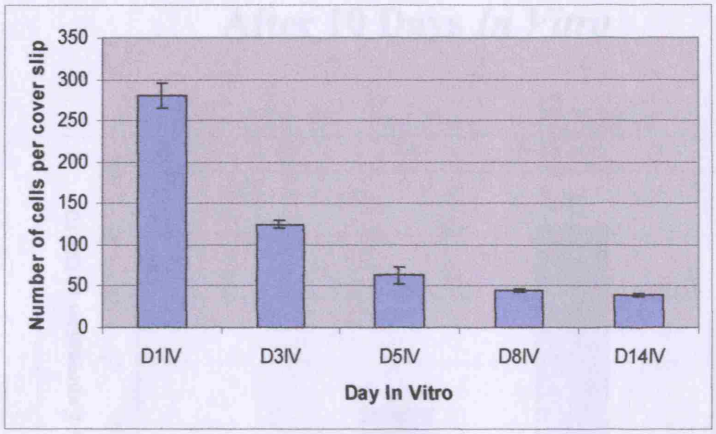


Fig 3.3
Dorsal horn neurones growing in culture stained with the pan neuronal marker PGP. Neurites are visible from day 7 in vitro onwards. By day 14 in vitro, the processes are fragmented (shown here as 'blebbing' of the neurites), and many of the cells no longer appear healthy. Scale bars 20 μm.

Survival of Dorsal Horn Neurones In Vitro



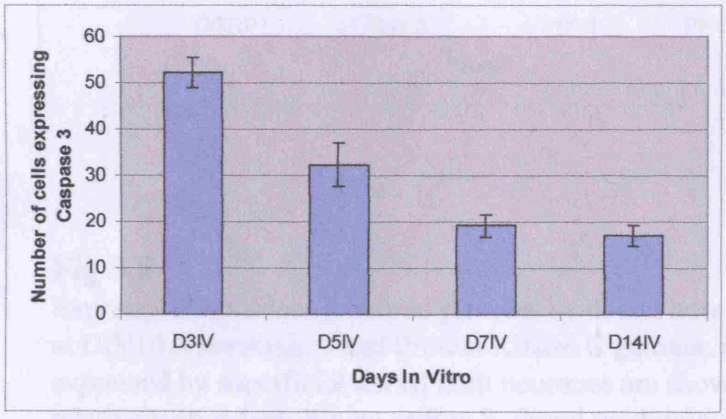


Fig 3.4
Cell counts over a 14 day period in vitro, showing the reproducible reduction in cell numbers per cover slip over time (cells stained by pan-neuronal marker, PGP). This high cell loss appears to be largely due to cell death (as shown by the expression of caspase 3) rather than cells simply floating off in to the medium.

Background Expression of Neurone Associated Proteins in Cultures of Dorsal Horn Neurones After 10 Days *In Vitro*

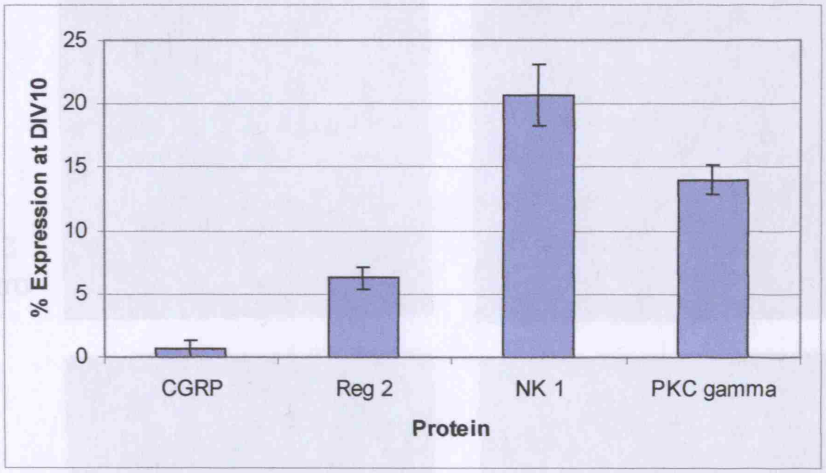


Fig 3.5

Expression of various neuronal proteins by dorsal horn neurones at DIV10.Neurokinin 1 and Protein Kinase C gamma, proteins expressed by superficial dorsal horn neurones are shown to be relatively abundant. Where as Reg 2, found predominantly in motor neurones, and Calcitonin Gene Related peptide, a marker of pre-synaptic C fibre terminals, are poorly expressed. n=3 cultures for each protein.

Expression of MAP2 & KCC2 in Dorsal Horn Neurones in Culture

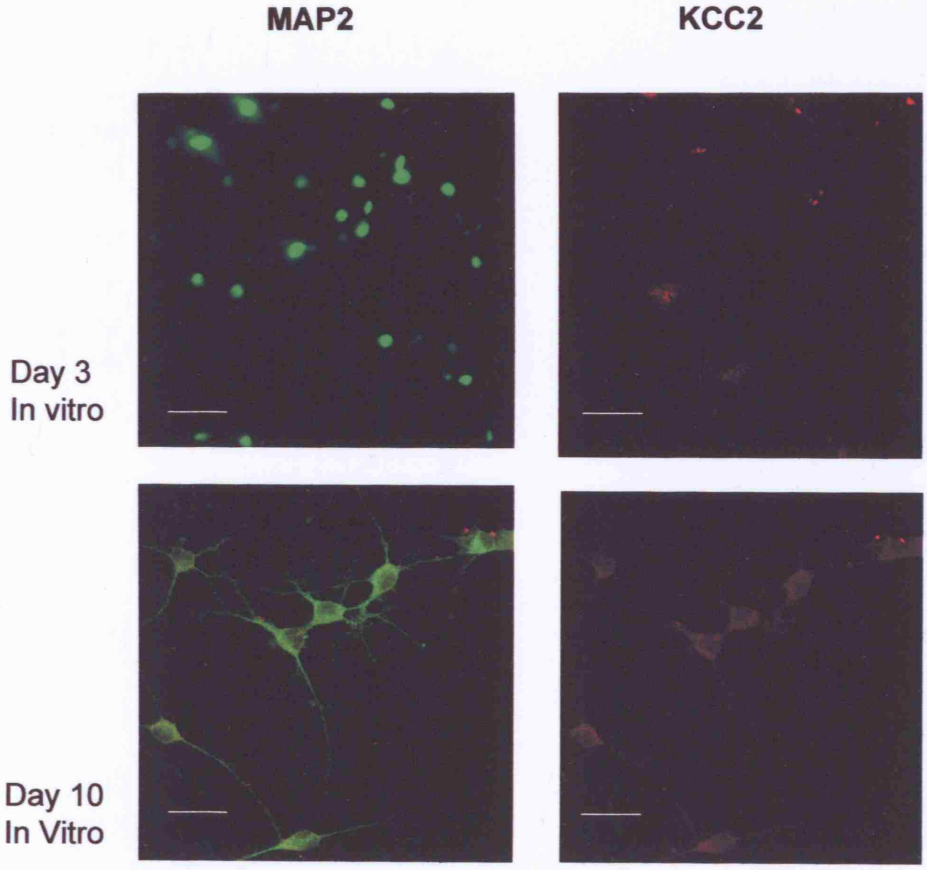


Fig 3.6
Confocal microscope images of MAP 2 (neuronal marker) and KCC2 expression at day 3 and day 10 in vitro. Expression at DIV 10 is shown to be greater than at DIV 3. Scale bars 20 μm.

Expression of KCC2 as a Percentage of MAP2 During *in vitro* Development

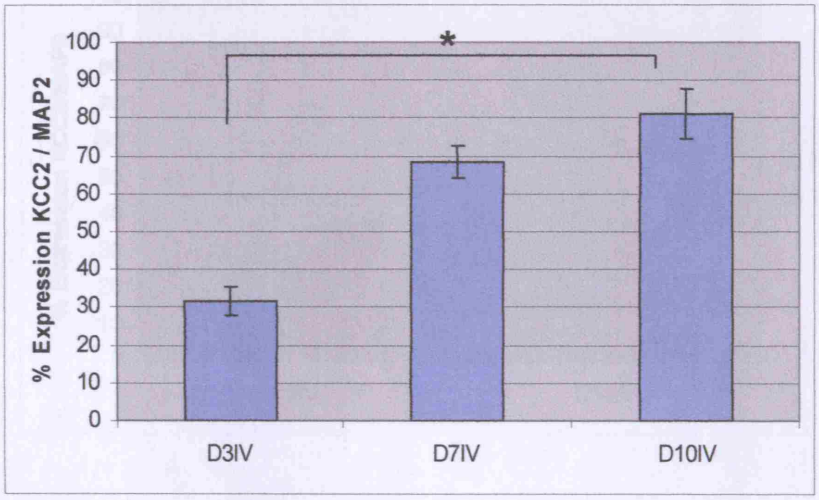


Figure 3.7
Graph showing increase in KCC2 expression as cells mature in-vitro (n=12). There is a significant rise between D3IV & D7IV ($P<0.05$, Student t test)

Effect of TTX on the Developmental Up-regulation of KCC2

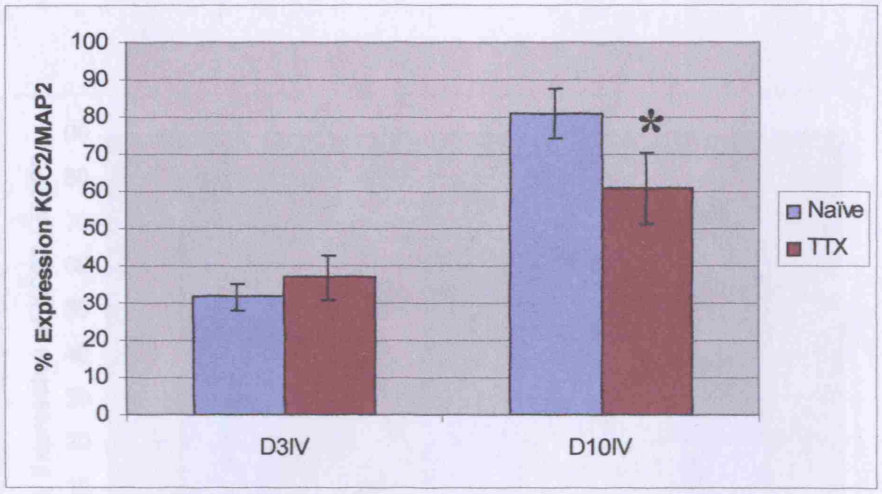


Figure 3.8
Expression of KCC2 as a percentage of the neuronal marker MAP2 at days 3 & 10 *in vitro*. Little difference is seen at DIV 3, but by day 10 there is a significant reduction in the expression of KCC2, when compared with naïve cells.

Effect of TTX & Receptor Inhibition on KCC2 Up-regulation at DIV10

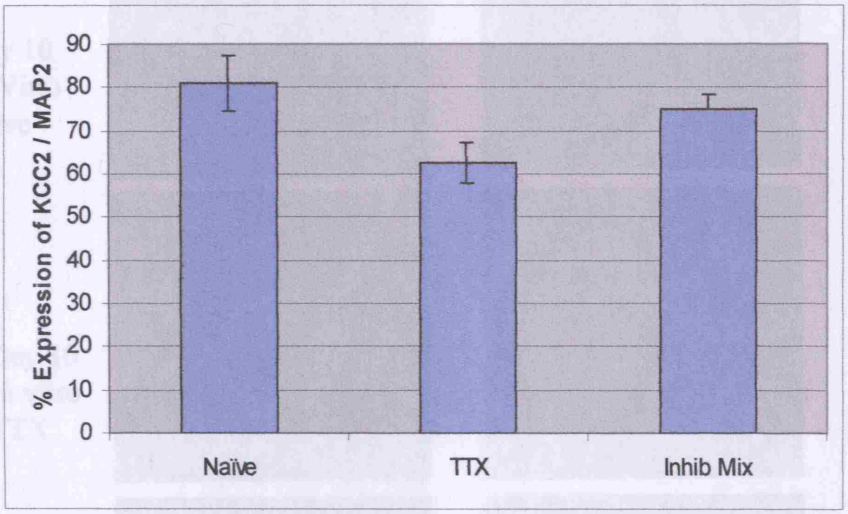


Figure 3.9
Action potential blockade using TTX shows a greater effect than ' receptor inhibitor cocktail' (blocking NMDA, non-NMDA, GABA & glycine). The effect of TTX is reproducible across n=12, and approaches statistical significance with P=0.07.

Expression of KCC2 in Cultured Dorsal Horn Neurones Following TTX Treatment & Receptor Inhibition

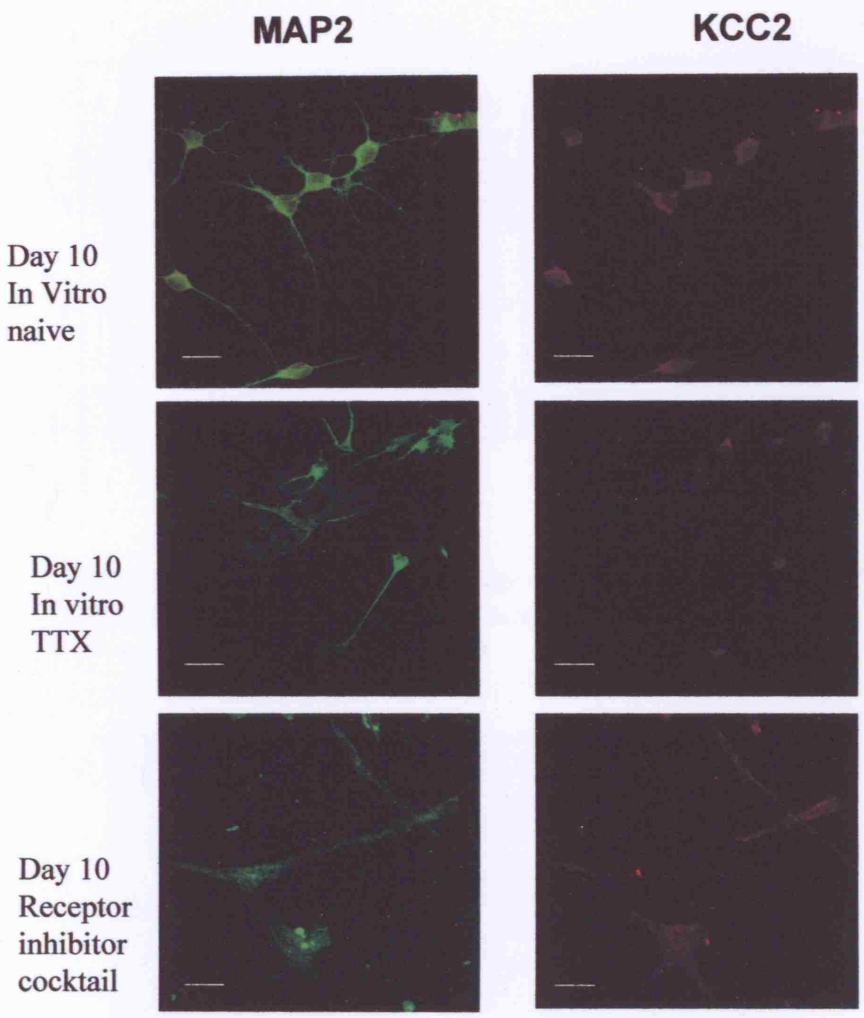
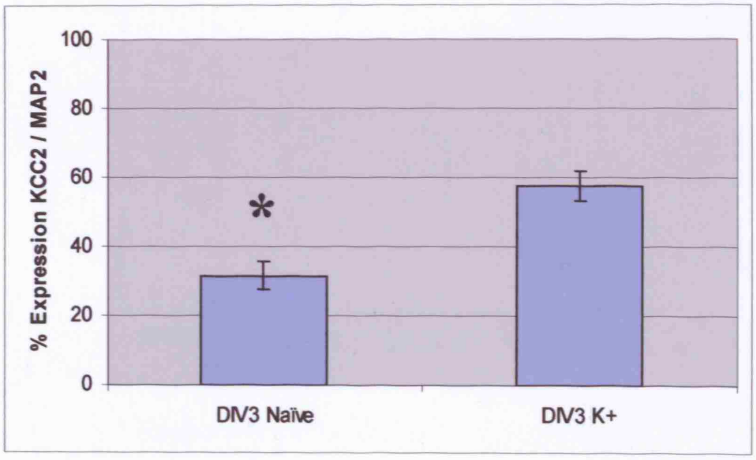


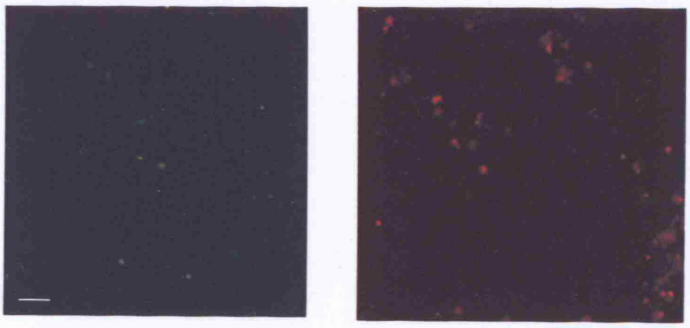
Fig 3.10

Confocal microscope images showing MAP 2 (neuronal marker) and KCC2 expression following the addition of the compounds listed to the culture medium. TTX is shown to reduce the expression of KCC2 at DIV 10, whereas the 'receptor inhibitor cocktail' does not have a significant effect. Scale bar 20 μm.

Expression of KCC2 in Cultured Dorsal Horn Neurones Following the Addition of Potassium to the Medium



Expression of KCC2 as a percentage of MAP2 positive cells at DIV3 in naïve cells and in cells treated with potassium

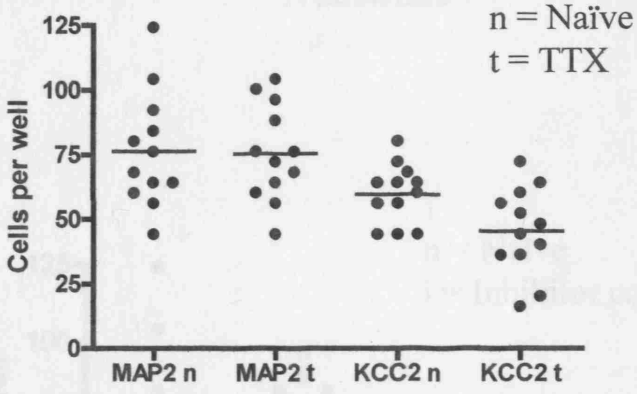


MAP2 **KCC2**

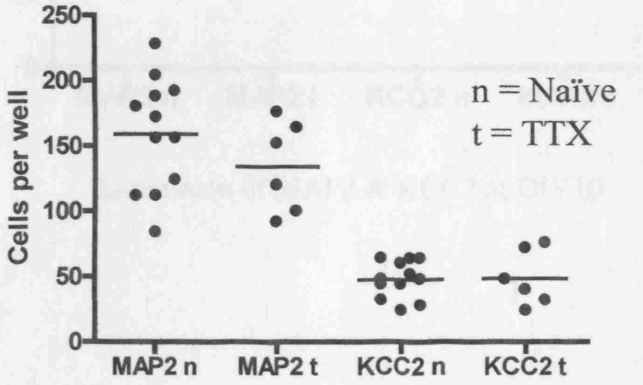
Fig 3.11

Expression of KCC2 is shown to be relatively higher in the cells treated with extra potassium ($P<0.05$, Student t test). Scale bars 10 μm.

The Effect of TTX on the Expression of KCC2 in Cultured Dorsal Horn Neurones



Expression of MAP2 & KCC2 at DIV10

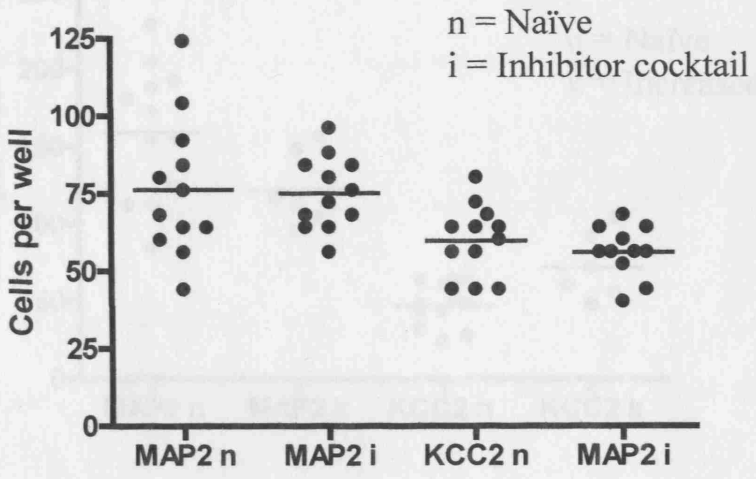


Expression of MAP2 & KCC2 at DIV 3

Fig 3.12

Scatter plots showing expression of MAP2 and KCC2 in dorsal horn neurone cultures grown with and without the addition of TTX to the medium. There is no significant difference in the expression of MAP2 (neuronal marker) with the addition of TTX at either age. TTX appears to have no effect on the expression of KCC2 at DIV3, but by DIV10 there is a significant reduction in the TTX treated cells ($P<0.05$).

The Effect of a 'Receptor Inhibitor Cocktail' on the Expression of MAP2 & KCC2 in Cultured Dorsal Horn Neurones

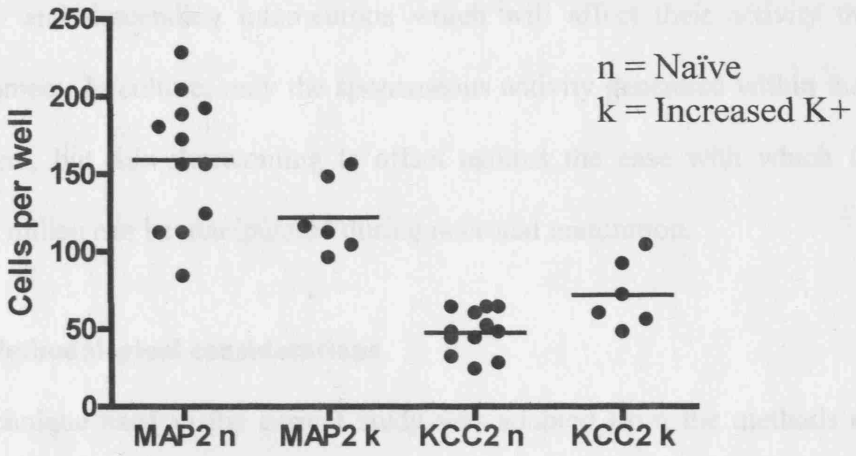


Expression of MAP2 & KCC2 at DIV10

Fig 3.13

Scatter plot showing the expression of MAP2 & KCC2 in dorsal horn neurones grown in culture with and without the addition of a 'receptor inhibitor cocktail' consisting of D-APV 20μM, NBQX 10 μM, Gabazine 10 μM, and strychnine 0.5 μM. The MAP2 (neuronal marker) levels remain constant with or without treatment, and no significant difference is seen in KCC2 expression.

The Effect of Increased Potassium Concentration in the Culture Medium on the Expression of KCC2 in Cultured Dorsal Horn Neurones



Expression of MAP2 & KCC2 at DIV3

Figure 3.14
Scatter plot showing the expression of MAP2 & KCC2 at DIV3. The addition of 10mM potassium caused cell death by one week *in vitro*. At DIV3 there is a slight (non-significant) reduction in MAP2 (neuronal marker) expression. However, the KCC2 expression at this stage is significantly higher than that of cells in untreated medium ($P<0.05$).

3.4 Discussion

In this chapter, we have provided direct evidence for the activity dependence of the developmental regulation of the cation chloride co-transporter KCC2. Dissociated neonatal dorsal horn neurons provide a useful model system in which to manipulate neuronal activity under controlled conditions. Cultured neurons are necessarily in an artificial situation, and within a network in the whole animal the situation is much more complicated; dorsal horn neurons receive input from primary afferents, as well as local and descending interneurons which will affect their activity throughout development. In culture, only the spontaneous activity generated within the cultures is present, but this shortcoming is offset against the ease with which the extra-cellular milieu can be manipulated during neuronal maturation.

3.4.1 Methodological considerations

The technique used in the current study was adapted from the methods of Jo and colleagues (Jo et al, 1998). In their original study, this group found the electrophysiological properties of the dissociated dorsal horn neurons recorded by patch clamping at day 6-10 *in vitro* to be similar to those of lamina I and II dorsal horn neurons in spinal cord slices (Jo et al, 1998). They also found that about half of the cultured neurons displayed glutamic acid decarboxylase (GAD)- like immunoreactivity, and / or met-enkephalin- like immunoreactivity; which is typical of dorsal horn neurons found in laminae II and III (Todd et al, 1992). Thus, they were able to conclude that their culture system contained essentially neurons from the most superficial laminae of the dorsal horn. In the present study, while being aware of the differences between the situations *in vitro* and *in vivo*, the morphology of the dissociated neurons was carefully studied over the first 2 weeks of

development. Here we found that the cultured cells began to extend processes at around day 4-5 *in vitro* (DIV), and could be seen to form connections from around days 5-7, consistent with other studies of cultivated spinal cord neurons (Nicola et al, 1992). The neurons continued to grow and form connections until DIV14, when they began to show signs of compromise, developing a fragmented appearance. Quantitative analysis was therefore conducted before this at days 3 and 10 *in vitro*. The numbers of dissociated neurons surviving in culture, per well, was counted over the same initial 2-week period. There was a pronounced drop in the first 5 days, before a stable population appeared to become established. Correspondingly, the specific apoptosis marker caspase III was highly expressed over the same time-period, suggesting that the cell-loss was not only due to cells failing to adhere to the culture wells. This is in keeping with the previously observed 'wave' of apoptosis shown to take place in the grey matter of the rat spinal cord shortly after birth (Lawson et al, 1997). The time course observed for apoptosis in sections of whole neonatal rat spinal cord (Lawson & Lowrie, 1998), is similar to that found in the cultured neurons used in the current study, occurring principally in the first 5 days of life. One limitation of the methods used here was the absence of a cell counter, consistent volumes of concentrated cell suspension prepared in a reproducible fashion were plated on to each cover slip, but cell density affects the survival and development of neurons in culture, and minor discrepancies may have occurred. In order to further qualify the population of cultured neurons, immunofluorescence studies were performed to assess the expression of various neuron-associated proteins. The expression of neurokinin 1 (NK1) and protein kinase C γ (PKCγ) was relatively abundant, in keeping with the phenotype of superficial dorsal horn neurons. NK1 expressing dorsal horn neurons are known to play an important role in

transmission and processing of nociceptive stimuli, they are particularly concentrated in laminae I & III of the superficial dorsal horn, where many of them represent projection neurons targeted by primary afferents (Labrakakis & McDermott, 2003). Protein kinase C (PKC) is thought to have a role in the sensitising of dorsal horn neurons in certain pain states, and mice lacking the PKCγ isoform show reduced pain after nerve injury. PKCγ is present in its highest concentration in lamina II of the spinal dorsal horn with some expression in laminae I & II where up to a third of cells co-express NK1 (Polgar et al, 1999). However, in contrast, reg2 (a motoneuron neurotrophic factor, Nishimune et al, 2000), and calcitonin gene related peptide (CGRP, a neuropeptide largely restricted to primary afferent neurons and motoneurons) were very poorly represented. Overall, this suggests that the majority of the surviving cells were likely to be dorsal horn neurons, as previously suggested by Jo et al, and further contributes to the validity of this cell preparation.

3.4.2 The expression of KCC2 throughout *in vitro* development

The expression of KCC2 in the dissociated dorsal horn neurons was upregulated over the first 10 days in vitro, in a manner analogous to that seen *in vivo* (see chapter 2). This similarity in KCC2 developmental profile both *in vivo* and *in vitro* has also been reported in cultures of hippocampal neurons (Ludwig et al, 2003). Interestingly, the onset of KCC2 upregulation in cultured neurons commenced as early as DIV 3, before neurons had established connections. This initiation of the KCC2 maturational process before the establishment of synaptic connections in isolated neuronal cells was also noted in the studies conducted on hippocampal tissue (Ludwig et al, 2003, Ganguly et al, 2001). Having established that the upregulation of KCC2 in untreated cells *in vitro* is sufficiently similar to that occurring in naïve whole animals, the model was used to investigate the impact of changes to the extra-cellular milieu.

Cells were studied at DIV3 & DIV10 in keeping with the time points used in earlier studies, however, it should be noted that DIV3 is an early time point after culturing neurons when their morphology is immature and expression of receptors / channels may be different to mature cells.

3.4.3 The impact of tetrodotoxin on KCC2 upregulation

Tetrodotoxin (TTX) is a potent marine toxin originally isolated from the Japanese puffer fish. It acts as a selective sodium channel blocker by binding to a receptor site on the extracellular side of the sodium channel, and preventing ions from flowing through it. This in turn prevents the cell concerned from being able to generate action potentials. TTX was added to the cell culture medium from DIV0, with each change of the solution. In accordance with previous reports, this did not affect the numbers of cells stained with the neuronal marker MAP2, nor did it appear to change the morphology of the cells (Colin-Le Brun et al, 2004). TTX has been shown to affect the development of functional GABAergic synapses in neonatal rat hippocampus by reducing locally generated spontaneous synaptic activity (Colin-Le Brun et al, 2004). A reduction in the upregulation of KCC2 in dorsal horn neurons may be expected if it is dependent on spontaneous neuronal activity. Patch clamp recordings from neonatal rat dorsal horn cells in culture have shown the presence of spontaneous action potential activity (Jo et al, 1998) and recordings from whole slices of neonatal rat spinal cord have shown that this intrinsic excitability of superficial dorsal horn neurons remains stable during early postnatal development (Baccei & Fitzgerald, 2005 in press).

Blockade of action potentials did not affect the small amount of KCC2 expression present at DIV3; however, the rapid increase between DIV3 and DIV10 was

significantly reduced. It therefore appears that reducing the electrical activity of the dissociated dorsal horn neurons has an impact on their postnatal development, suggesting that the maturation of GABA and glycinergic transmission is indeed activity dependent.

3.4.4 The impact of receptor inhibition on KCC2 upregulation

Having established the importance of electrical activity on the developmental regulation of chloride transport, the experiment was repeated with the addition of a 'receptor inhibitor cocktail' to the medium. The aim of this was to eliminate transmission via GABA, glycine and glutamate receptors during the immediate neonatal period. As the cultured cells consist of a mixture of excitatory and inhibitory neurons from the superficial dorsal horn, all three of these transmitters are likely to be represented (Todd, 2001). Which transmitters (if any) affect the upregulation of KCC2 remains highly controversial. In hippocampal neurons, Ganguly and Ludwig agree that glutamatergic transmission has no impact, but whilst Ganguly finds GABAergic signalling to be essential, this is not reproduced by Ludwig (Ganguly et al, 2001; Ludwig et al, 2003). In auditory neurons, Shibata suggests that all three transmitters play a role in driving KCC2 expression (Shibata et al, 2004), whereas in the turtle retina, Sernagor demonstrates a role for GABA alone (Sernagor et al, 2005). In the present study there was no significant effect following blockade of all three transmitter systems with only a small non-significant reduction in expression at DIV 10. Our findings suggest that the activity required to drive the upregulation of KCC2 is not critically dependent on any of the principle synaptic neurotransmitters present. Despite regular reapplication of the inhibitors (with each change of medium), it is also still possible that blockade was not complete or uninterrupted for all three transmitters. In particular, immature glycine receptors,

expressing the α_2 subunit, are known to be relatively less susceptible to strychnine (Aguayo et al, 2004), however observations from our own laboratory suggest that functional blockade of glycinergic transmission by strychnine is possible in the neonatal period (ML Baccei, unpublished data). Although it is also conceivable that transmitters other than the classical three systems inhibited (such as substance P, 5-HT) may be active synaptically, the fact that KCC2 up-regulation begins *in vitro* ahead of synapse formation is against a synaptic mechanism.

3.4.5 The impact of supra-physiological potassium concentration on KCC2 upregulation

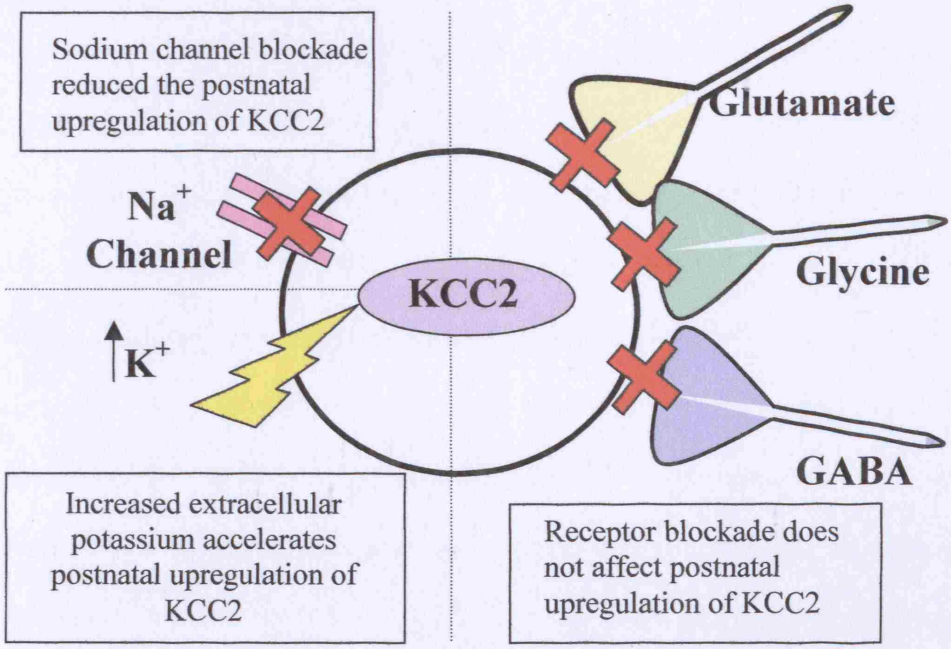
Having demonstrated that spike activity, but not synaptic, receptor mediated transmission was essential for the maturation of KCC2 levels, elevated potassium in the medium was used to create increased neuronal excitation. High concentrations of extracellular potassium increase the resting membrane potential of neuronal cells, lowering the threshold needed to initiate spiking. Unfortunately, in this case, the treated cultured cells were reduced in number compared to controls, and by DIV10, the cells were in such poor condition that they could not be used. However, at DIV3 the expression of KCC2 was almost twice that of the control neurons, suggesting that the upregulation had been accelerated by the addition of potassium. Addition of increased potassium to the culture medium has previously been shown to cause a two fold increase in endogenous GABA release from fetal mouse striatal neurons (Weiss et al, 1986), suggesting that at least an element of GABAergic synapse development is activity dependent in the same manner. The accelerated early upregulation of KCC2 occurring in the face of elevated potassium is occurring before the advent of synapse formation *in vitro*. This suggests that the electrical activity involved may be an intrinsic property of the developing dorsal horn neurons.

3.4.6 Conclusion

The results discussed above demonstrate that KCC2 upregulation is accelerated by increased extracellular potassium concentrations and inhibited by the addition of TTX to the culture medium. This indicates a dependence of the process on cellular electrical activity. However, a 'cocktail of inhibitors' designed to eliminate synaptic activity by the main transmitters known to be present, failed to produce a significant effect. Although the aim here was to eliminate all classical synaptic activity, there is some evidence that chronic blockade of AMPA / kainite (excitatory signalling) or glycine & GABA receptors (inhibitory signalling) produce opposite effects on the electrical activity of organotypic cultures of mature spinal cord (Galante et al, 2000). This would therefore make it possible that the agents used here 'cancelled each other out'. However, assuming that the inhibitors were effective, this would otherwise suggest either that the process is independent of synaptic activity, or that another 'unblocked' substance was involved. For example, previous work on cultured neonatal dorsal horn neurons has revealed that ATP produced both pre- and postsynaptic effects on GABAergic transmission via P2X2 receptors (Hugel & Schlichter, 2000). There is also a possibility that other modulating factors, which would have a regulatory role on synaptic transmission *in vivo* are not reproduced in vitro, examples include 5α reduced neurosteroids (Keller et al, 2004) and oxytocin (Jo et al, 1998). Interestingly, our results, in keeping with the work of others (Ludwig et al, 2003; Ganguly et al, 2001) showed some upregulation of KCC2 before the stage of synapse formation *in vitro*, making a conventional synaptic process unlikely to be the sole trigger. GABA is known to operate before the advent of synapse formation, and to have a trophic role in neuronal maturation (Represa & Ben-Ari, 2005). This developmental property of GABA may not be adequately reproduced in

certain culture protocols, as it involves cooperation with numerous other molecules, and may therefore account for some of the inconsistencies between reported results (Ludwig et al, 2003; Ganguly et al, 2001). The trophic effect of GABA in early development has also been shown to be quantitatively different between distinct parts of the nervous system (Represa & Ben-Ari, 2005), possibly accounting for differences found between hippocampal neurons and dorsal horn neurons. Spontaneously occurring neuronal activity occurring in developing rat hippocampal neurons, has been shown to be important in the formation of functional GABAergic synapses; affecting the levels of immunoreactivity for GABA and its synthetic enzymes and at least some of this 'activity' can be shown to be TTX dependent (Colin-Le Brun et al, 2004). Thus, spontaneous electrical activity in neonatal dorsal horn cells could be postulated to drive the upregulation of KCC2. Although spontaneous rhythmic oscillatory behaviour of neurons has been studied extensively in other areas of the CNS, little work has focussed on the dorsal horn of the spinal cord. A recent study of neonatal rat substantia gelatinosa neurons in culture demonstrated the presence of potassium evoked oscillatory activity, which was only partly dependent on glutamate, GABA or glycinergic transmission, but significantly reduced by TTX or by blockers of gap junction (Asghar et al, 2005). Other reports have also suggested that bursting in spinal cultures is mainly based on the intrinsic spiking of certain neurons (Darbon et al, 2002), making this an attractive hypothesis for the drive behind the upregulation of KCC2. Indeed, observations from patch clamp recordings in our own laboratory have shown a significant degree of spontaneous activity in neonatal superficial dorsal horn neurons, (Baccei & Fitzgerald, 2005). This activity appears to be highly dependent on action potentials within the local circuit in neonatal dorsal horn neurons, as TTX application has a

large effect on spontaneous excitatory postsynaptic current frequency compared to the situation in adult dorsal horn (Baccei et al, 2003). Slack and colleagues have also demonstrated the presence of spontaneous activity, in the first week *in vitro,* in cultured neonatal dorsal horn neurons, using the same protocol as the current study (Sarah Slack, PhD Thesis, 2003). However, in contrast, studies carried out in neonatal hippocampus cultures, have not consistently shown a reduction in KCC2 upregulation following the application of TTX. It is possible that this reflects differences in the nature of spontaneously occurring neuronal activity between the neonatal hippocampus and dorsal horn. The hippocampal activity is characterised by the presence of giant depolarising potentials (Sipila et al, 2005), which are much less abundant in neonatal superficial dorsal horn (ML Baccei, unpublished observations). In conclusion, we have shown that the postnatal upregulation of KCC2 is dependent on electrical activity as exemplified by the effects of TTX and increased extracellular potassium; however, as this was unaffected by receptor blockade it appears likely that the upregulation is driven by spontaneous intrinsic activity of young dorsal horn neurons (see below).



Chapter Four

Developmental Aspects of Pain Behaviour Following Intrathecal Administration of GABA, Glycine and Their Antagonists

4.1 Introduction

Inhibitory transmission plays an important role in normal pain processing in the mature nervous system, and its absence has been implicated in the aetiology of certain pain states (Dickenson et al, 1997). The neonatal nervous system is characterised by a relative lack of inhibition (Fitzgerald, 1999), yet there have been few functional studies of the development of inhibition in the spinal cord. *In vivo* studies have demonstrated that the cutaneous receptive fields of neonatal rat dorsal horn neurons are larger when compared to those of mature animals (Fitzgerald & Torsney, 2002); and that cutaneous flexion withdrawal reflexes are exaggerated both in rat pups and human infants (Andrews & Fitzgerald, 1994). Both of these observations are consistent with immature inhibitory signalling at the level of the spinal cord dorsal horn.

Local blockade of glycinergic and / or GABAergic neurotransmission in the mature rat spinal cord causes hypersensitivity to noxious stimulation, analogous to the state found in neonatal animals. Intrathecal administration of strychnine (glycine antagonist) or bicuculline ($GABA_A$ antagonist) produces measurable tactile allodynia in adult rats, and is sometimes used as a model of chronic pain. Furthermore, co-administration of strychnine and bicuculline has revealed a cooperative (supra-additive) effect (Loomis et al, 2001), suggesting that combined loss of GABA and glycinergic signalling following neuronal injury may be additive in the generation of neuropathic pain. Strychnine dependent allodynia has been demonstrated to be dose dependent and immediately reversible by the application of glycine, with spontaneous recovery otherwise occurring by 30 minutes following a single injection (Sherman & Loomis, 1995). There are no cardiovascular, motor, or

electroencephalographic changes associated the administration of intrathecal glycine, making a local spinal site of action more likely. A central site of action for the sensitisation is further supported by the work of John et al, who showed that the effect of intrathecal bicuculline in enhancing pain behaviour during the formalin test is independent of the degree of peripheral inflammation (John et al, 1998). Further work on strychnine and bicuculline induced hyperalgesia has shown it to be selectively reversed by the NMDA antagonist MK801 (Onaka et al, 1996; Yamamoto et al, 1993). As MK801 had no effect on sham operated animals or controls, it implies that the induced allodynia was mediated through a pathway involving NMDA signalling. Conversely, it has been shown that continuous intrathecal infusion of glycine is sufficient to prevent the mechanical hyperalgesia associated with sciatic nerve ligation (Huang et al, 2000); and equally, a single intrathecal injection of GABA given during the critical, early, induction phase of neuropathic pain is sufficient to prevent its development (Eaton et al, 1999). Thus, in mature animals, $GABA_A$ and glycine antagonists produce sensitisation of naïve rats, when injected intrathecally, whereas this sensitisation may be reversed by application of GABA and glycine themselves, with evidence suggesting that this is a local effect at the level of the spinal cord.

Inhibition may be evoked phasically by external stimuli or be present tonically. A degree of GABA and glycinergic inhibitory tone has been shown to be present in the adult spinal and medullary dorsal horn (Cronin et al, 2004; Ishikawa et al, 2000). Cronin et al used C-Fos activity to map the distribution of tonically inhibited cells in the healthy rat spinal cord. They found a marked increase in C-Fos labelling following the intravenous administration of strychnine or picrotoxin, with a marked

difference in the regional distribution of labelling between the compounds. Both drugs produced labelling in the deeper laminae, but picrotoxin induced strong Fos-like immunoreactivity in the superficial dorsal horn, whereas strychnine did not (Cronin et al, 2004). Interestingly, although both blockade of $GABA_A$ and glycine transmission provoke hypersensitivity to low intensity (non-noxious) stimuli (Sorkin et al, 1998); $GABA_A$ antagonists also elevate the response to normally noxious inputs (implying a role at the level of the superficial dorsal horn), but glycine antagonists do not (Sherman & Loomis, 1996). This may relate to differences in the anatomical distribution of GABAergic and glycinergic tone in the naïve spinal cord (Cronin et al, 2004).

As previously discussed in chapter 2, GABA and glycinergic signalling in the mammalian CNS is subject to striking developmental regulation. Experiments conducted *in vitro*, have revealed a clear excitatory role for GABA in immature neurons (Wang et al, 1994; Ben-Ari et al, 2002), attributed to the relatively high intracellular chloride concentration in young neurons, which in turn is due to the lack of its active extrusion by the cation co-transporter KCC2 (Rivera et al, 1999). In spinal cord slices taken from adult rats, blockade of KCC2 with [(dihydroindenyl)oxy]alkanoic acid (DIOA), caused an increase in $[Ca^{2+}]$ in 30% of lamina I neurons (Coull et al, 2003), confirming the role of the co-transporter in determining the nature of GABAergic signalling. Developmental upregulation of KCC2 has been demonstrated in a number of CNS regions, and postnatal changes at the level of the spinal cord have been demonstrated in chapter 2 of this thesis. Patch clamp recordings have also shown that GABA can be depolarising in the superficial dorsal horn in the first days of life (Baccei & Fitzgerald). However, evidence for a

developmental 'switch' in polarity of GABAergic transmission on normal neonatal CNS procession *in vivo* has not been examined.

In this chapter, intrathecal injections of GABA, glycine and their antagonists were administered to rats ages P3 and P21, and the impact on their cutaneous withdrawal thresholds was measured. This was performed according to a modification of the method of Hylden & Wilcox. This technique for local delivery of drugs to the spinal cord, by single intrathecal injection has been validated by the use of radiolabelled morphine, which was found not be present in significant quantities in the midbrain or forebrain, despite producing the expected local functional analgesia (Hylden & Wilcox, 1980). As the protocol was devised for use in adult mice, validation was repeated in infant (P3) rats, with experiments being repeated using radiolabelled drug in order to assess the extent of any to rostral spread of the compounds.

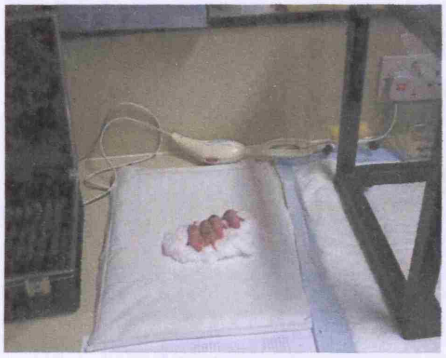
4.2 Materials and methods

Sprague Dawley rats of both sexes were used. These were bred by the Central Animal Facility, University College London, and were kept in artificial lighting on a 12:12h light cycle with the temperature maintained at a constant 21°C. Food and water were given *ad libitum* and all procedures were carried out in accordance with the United Kingdom Animal Procedure Act 1986. P3 and P10 rat pups were housed with their mother and littermates. P21 animals were housed independently in groups of 6. All behavioural experiments were carried out with a specific set of von Frey hairs, which were calibrated prior to the study. This was done by applying the filaments 3 times each to a non-slip surface on a set of weighing scales and recording the force produced (see fig 4.1a). This was found to be reproducible in the hands of the investigator, over the range required.

4.2.1 Behavioural Investigations

Experiments were carried out on rat-pups aged P3, P10, and P21. All behavioural investigations took place in the same, temperature controlled, room. Animals were habituated to the room and the testing apparatus, on two consecutive days immediately prior to drug administration for an hour (30 minutes in the case of P3 animals because of the risk of hypothermia). Mechanical withdrawal thresholds were measured by application of von Frey hairs (vFh) to the plantar surface of the paw, while the animals were standing on an elevated wire mesh. These numbered nylon monofilaments of increasing rigidity were applied until a flexion withdrawal was evoked, and the lowest numbered vFh that elicited a positive withdrawal of the hindpaw was recorded. Baseline thresholds for paw withdrawal were measured on two separate occasions for each animal, prior to drug administration. Five

applications of each vFh were made and the threshold was considered to be the hair that elicited a withdrawal response in 60% or more of applications.



P3 rats on heat pad

P21 animal habituating

Animals were anaesthetised with 4% halothane in O_2 in a gas box and maintained at 1.5% halothane via a mask. Intrathecal administration of GABA, glycine and their antagonists (as well as saline controls) were performed, using a 10 µl Hamilton micro syringes (26S gauge, model 801RN, Hamilton Bonaduz AG, Switzerland). Total volumes of drug and saline were adjusted according to the animal's age, with P21 animals receiving 10µl, P10 animals receiving 5µl, and P3 animals receiving 3µl. The investigator administering the drug was blinded as to the whether the solution was active drug or saline control.

Doses of the drugs were calculated based on those previously used in the literature, adjusted according to the animal's weight, as there appears to be little experience of neonatal intrathecal dosage. The converted doses were as follows:

- GABA - 6 nanograms per gram (Eaton et al, 1999)
- Glycine - 2 nanograms per gram (Sherman et al, 1995)

- Bicucculine - 10 nanograms per gram (Loomis et al, 2001)
- Strychnine - 165 nanograms per gram (Loomis et al, 2001)
- Gabazine – 1 nanogram per gram (Boissard et al, 2002)

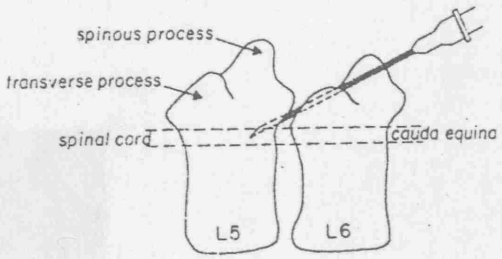


Diagram of the anatomical landmarks for IT injection, from Hylden & Wilcox (1980)

Injections were carried out around the level of L5-L6 (near to where the spinal cord ends and the cauda-equina begins), as per the method of Hylden and Wilcox (1980). The animal was held firmly by the pelvic girdle in one hand while the syringe was held in the other at an angle of about 20 degrees above the vertebral column. The needle was inserted into the groove between the transverse and spinous processes. The angle of the syringe was then reduced to 10 degrees and the needle was moved forward a short distance inside the vertebral column. The drug solution was slowly injected and the needle rotated on withdrawal.

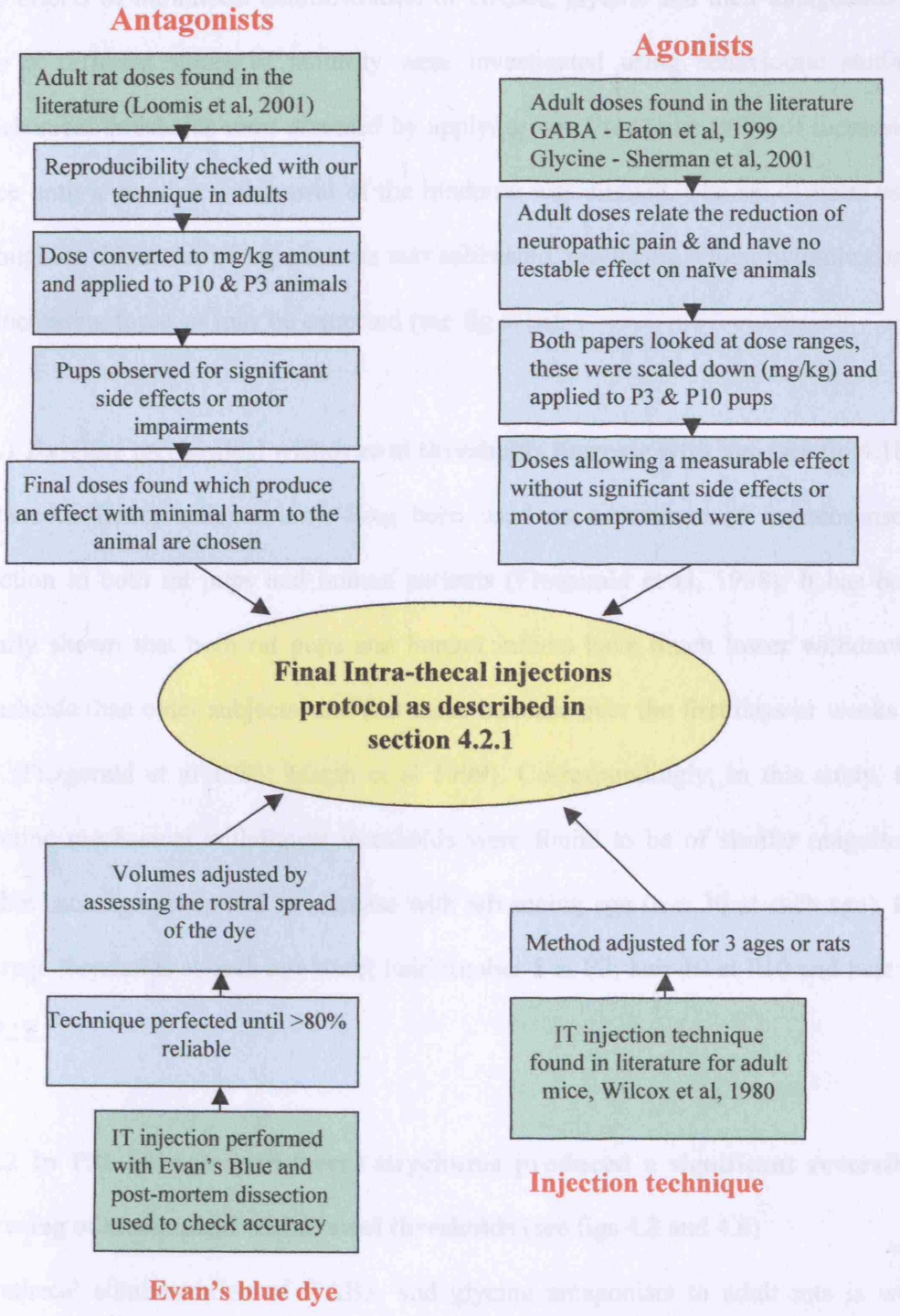
The animals were allowed to recover from the anaesthetic and von Frey hair testing of mechanical withdrawal thresholds was commenced at 5 minutes. Measurements were made every 5 minutes for the first 30 minutes and every 10 minutes between 30 minutes and 60 minutes post injection. After the testing period of one hour, the animals were sacrificed and dissected in order to assess the accuracy of the injection site. Animals with any overt motor impairment were discarded from the study. In

some cases, the accuracy of the injection site was further checked by the introduction of Evans Blue dye in the drug solution, this allowed post-mortem visualisation of the injected solution. Data from Evans Blue injections were not included in any analysis presented in this thesis, as the effect of the dye itself when administered intrathecally are not known.

P3 rat pup following IT injection of Evan's Blue dye (post mortem photo).

4.2.2 Visualisation of Gabazine Binding Sites

Neonatal rat pups (P3) were lightly anaesthetised with 4% halothane/O_2 in a gas box and maintained at 1.5% halothane via a gas mask. A 10µl Hamilton syringe (26S gauge, model 801 RN, Hamilton Bonaduz AG, Switzerland) with a fixed needle was filled with 0.9% sterile saline with 2.2µCi of ^{3}[H]Gabazine (Specific activity = 55.3Ci/mmol) Perkin Elmer Boston, MA. The needle tip was gently advanced below the dura and the compound was injected under direct vision. The syringe was slowly withdrawn and at 10min post injection the animals were terminally anaesthetised with Euthetal. Tissue was mounted on a cryostat chuck and snap frozen on dry ice. Frozen transverse sections (30µm) were thaw mounted on superfrost plus microscope slides dried overnight and exposed to a phosphorimage screen for 10 days. The phosphoimage screen was then scanned using a Typhoon Phosphoimager (Amersham, UK).



Development of a technique for intra-thecal injections of GABA, glycine and their antagonists in rat pups

4.3 Results

The effects of intrathecal administration of GABA, glycine and their antagonists *in vivo* at different stages of maturity were investigated using behavioural studies. Mechanical thresholds were assessed by applying von Frey hairs (vFh) of increasing force until a positive withdrawal of the hindpaw was elicited. The set of hairs used throughout this series of experiments was calibrated, producing a logarhythmic curve of increasing force, as may be expected (see fig 4.1a).

4.3.1 Baseline mechanical withdrawal thresholds increase with age (see fig 4.1b)

Cutaneous flexor reflexes have long been used as a measure of somatosensory function in both rat pups and human patients (Fitzgerald et al, 1988). It has been clearly shown that both rat pups and human infants have much lower withdrawal thresholds than older subjects, and that these increase over the first days or weeks of life (Fitzgerald et al 1988; Marsh et al 1999). Correspondingly, in this study, the baseline mechanical withdrawal thresholds were found to be of similar magnitude within each age group and to increase with advancing age (n = 36 at each age), the average thresholds at each age were; hair number 5 at P3, hair 10 at P10 and hair 18 at P21.

4.3.2 In P21 animals intrathecal strychnine produced a significant reversible lowering of mechanical withdrawal thresholds (see figs 4.2 and 4.6)

Intrathecal administration of GABA and glycine antagonists to adult rats is well known to produce mechanical sensitisation (Loomis et al, 2001), and indeed this effect is often used as a model of tactile allodynia. Here we have shown a significant reversible, sensitisation in P21 animals following intrathecal administration of

strychnine (n=12 in each group, P<0.05, ANOVA). The effect was maximal at 5-10 minutes post injection, consisting of a difference of 14 vFh units, with recovery occurring by 30 minutes.

4.3.3 In P21 animals intrathecal bicuculline also a produced a significant reversible lowering of mechanical withdrawal thresholds (see figs 4.3 and 4.2)

Intrathecal bicuculline produced a similar effect to that of strychnine, causing a significant maximum reduction in mechanical withdrawal threshold of 6 vFh units by 5 minutes post injection (n=12 in each group, P<0.05, ANOVA). Once again full recovery occurred by 30 minutes. This data is consistent with the effects of bicuculline previously described in the literature.

4.3.4 In P3 rat pups intrathecal strychnine produced a significant, reversible INCREASE in mechanical withdrawal thresholds (see fig 4.2 and 4.6)

The glycine antagonist strychnine has been shown to have the opposite effect to that seen in mature rats when applied intrathecally to P3 pups. The classically low withdrawal thresholds of the young animals are actually increased (desensitised) by the application of strychnine. The effect achieved significance by 5 minutes post application, reaching a difference of 4 vFh units (n=12 in each group, P<0.05, ANOVA), and recovery was complete by 35 minutes. This data confirms that glycine antagonists have the opposite (inhibitory) effect when administered to an intact rat pup at P3, and thus suggests the potential for an excitatory role for glycine in the in immature animal *in vivo*.

4.3.5 In P3 rat pups intrathecal bicuculline also produced a significant, reversible INCREASE in mechanical withdrawal thresholds (see fig 4.3 and 4.2)

The $GABA_A$ antagonist bicuculline produced a similar effect to that of strychnine in P3 rat pups, causing a reduced sensitivity to mechanical stimuli in a manner opposite to the effect in mature animals. The effect was maximum at 5 minutes, producing a statistically significant difference of 4 vFh units ($n=12$ in each group, $P<0.05$, ANOVA), with recovery by 30 minutes post injection

4.3.6 GABA and glycine have no measurable effect when administered intrathecally to P21 rats (see figs 4.4, 4.5 & 4.7)

The mechanical withdrawal thresholds of P21 rats were unaffected by the application of GABA or glycine intrathecally ($n=12$ in both groups). In the case of untreated mature rats, application of von Frey hairs up to the force required to physically lift the paw did not reliably produce a withdrawal, as their baseline sensitivity is low. This would make it difficult to measure a further reduction in threshold in response to the administration of inhibitory transmitters. Another possible reason for the effect being undetectable in the naïve adult rat is likely to be the existence of a degree of GABAergic tone, such that the addition of a further aliquot of GABA would be of little significance.

4.3.7 In P3 pups intrathecal administration of GABA produces a significant reversible LOWERING of mechanical withdrawal thresholds (see fig 4.4 & 4.7)

GABA was shown to produce a significant reversible sensitisation of mechanical withdrawal thresholds (excitation) with a difference of 3 vFh units maximal at 5 minutes post treatment, with recovery complete by one hour ($n=12$ in each group,

P<0.05, ANOVA). This GABAergic excitation has not previously been demonstrated *in vivo* to the best of our knowledge.

4.3.8 In P3 pups intrathecal administration of glycine produces a reversible LOWERING of mechanical withdrawal thresholds, but this fails to reach statistical significance (see fig 4.5 & 4.7)

Glycine produced a similar effect to that of GABA, over a parallel time course, however the effect was of a lesser magnitude (1.5 vFh units) and as such failed to reach statistical significance (n=12 in each group). It does however constitute a notable difference from the lack of glycine effect at P21.

4.3.9 Gabazine reproduces the increase in mechanical withdrawal thresholds produced by bicuculline in P3 animals (see fig 4.8)

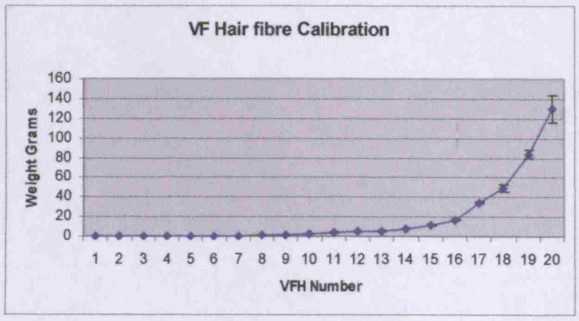
Despite being a commonly used $GABA_A$ receptor antagonist in the field of neuroscience, bicuculline has recently been shown to have some blocking activity at voltage gated potassium channels in the rodent CNS (Druzin et al, 2004). Later experiments were therefore carried out using gabazine (a relatively pure $GABA_A$ antagonist). Gabazine (1ng/g) caused the same significant, reversible increase in mechanical withdrawal thresholds in P3 rats as had been observed with bicuculline (10ng/g) (n=8, P<0.05, ANOVA). The effects of gabazine in mature animals are well documented (Boissard et al, 2002) and are equivalent to those of bicuculline.

4.3.10 Intrathecal administration of radiolabelled gabazine to P3 rats demonstrates that the drug does not spread significantly more rostrally than the lumbar region (see fig 4.9)

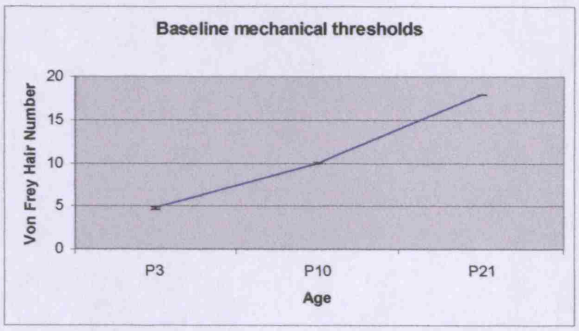
In order to establish that the intrathecally-administered drugs were exerting their effect locally, at the level of the lumbar spinal cord, radiolabelled gabazine was injected into P3 rats in the same volume (3µl), and the spread of the drug was assessed. Radio-gabazine was clearly detectable in the lumbar spinal cord, with minimal spread to the thoracic level, and no detectable drug further rostrally (n=4). This confirms that the effects described are not due to cephalic spread of the drugs, but to a local action at the level of the lumbar spinal cord.

Calibration of Von Frey hairs, Baseline Mechanical Thresholds by Age & Controls

a



b



c

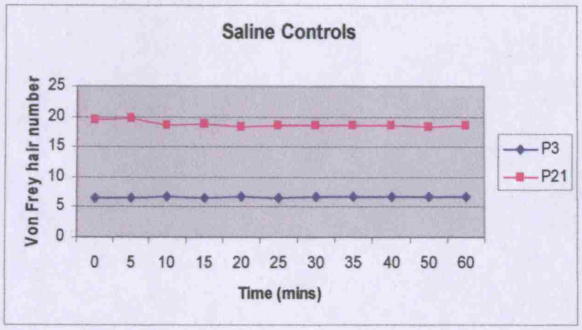


Fig 4.1

a. Calibration of Von Frey hairs used for all behavioural experiments, each hair was applied to a balance on three separate occasions and the mean taken.
b. Baseline mechanical thresholds for naïve rats of different ages (n=36 at each age)
c. Saline controls at P3 & P21 (n=24 at each age)

Intrathecal Administration of GABA & Glycine Antagonists

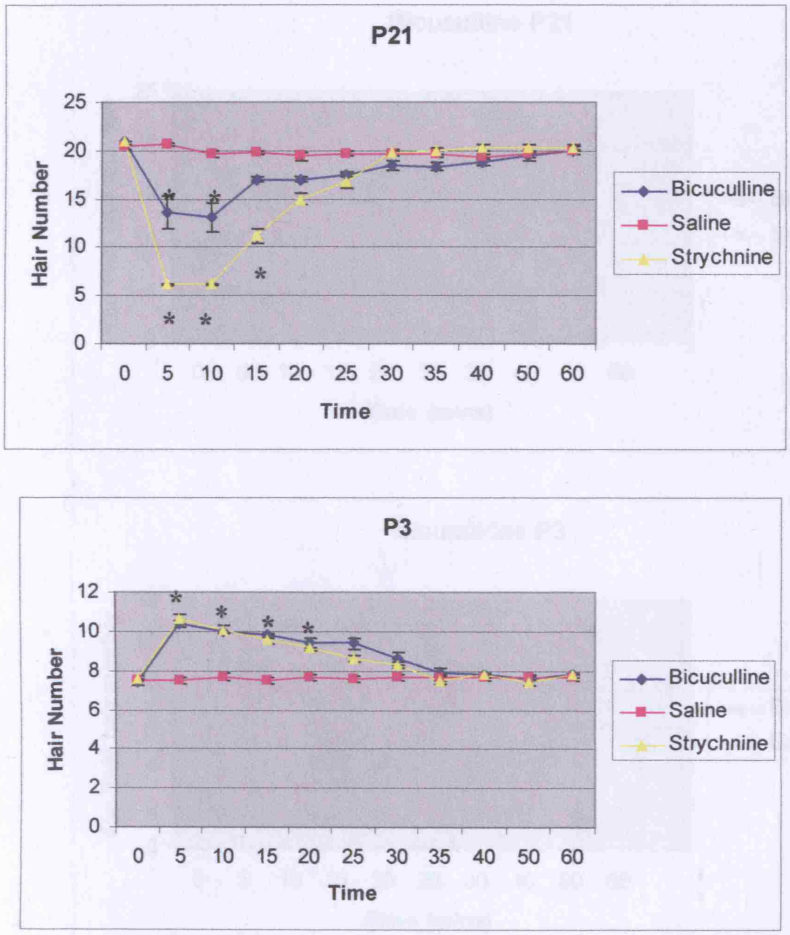


Fig 4.2

Mechanical thresholds following intrathecal administration of bicucculine, strychnine, and saline control to rats aged P3 & P21. n=12 in each group, and * $P<0.05$ (ANOVA). P21 animals are shown to be significantly sensitised by strychnine & bicucculine for 15 minutes following injection, with recovery by 30 minutes. P3 pups have a **rise** in their thresholds for 20 minutes post injection, with reversal of the effect by 35 minutes.

Intrathecal Administration of Bicuculline

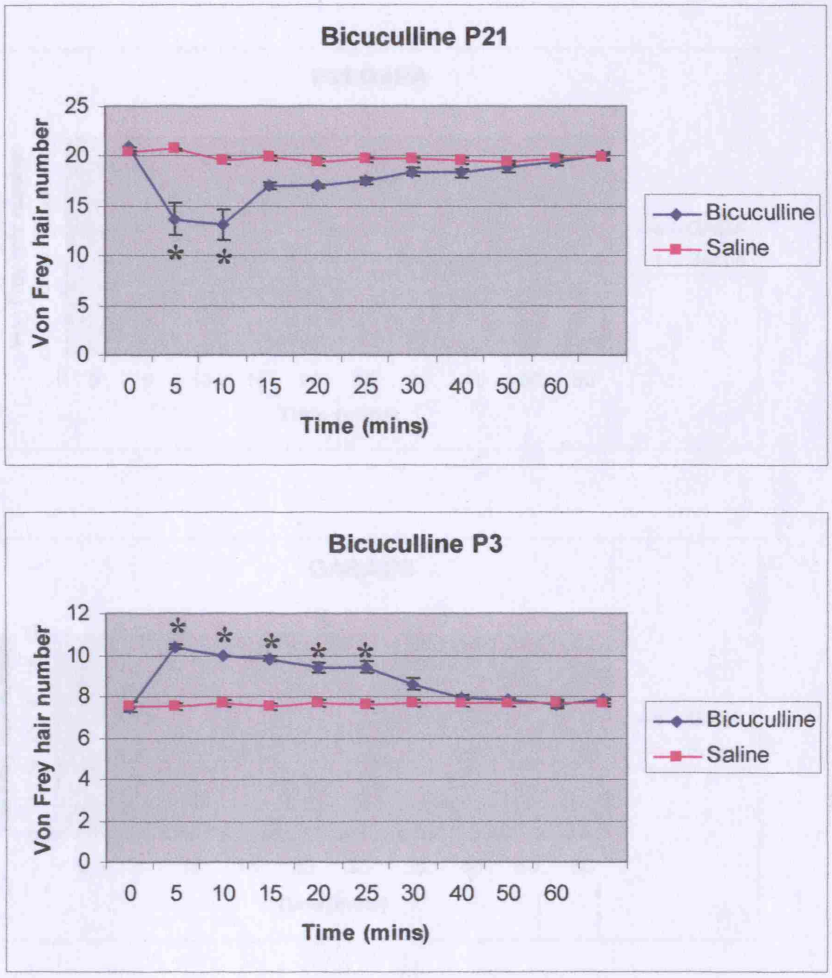


Fig 4.3
Mechanical thresholds following intrathecal administration of bicuculline and saline vehicle control to rats aged P3 and P21. n = 12 in each group, * P <0.05. P21 animals are significantly sensitised for 10 minutes following injection, whereas P3 animals show an opposite effect with a RISE in their thresholds for 25 minutes post injection.

Intrathecal Administration of GABA

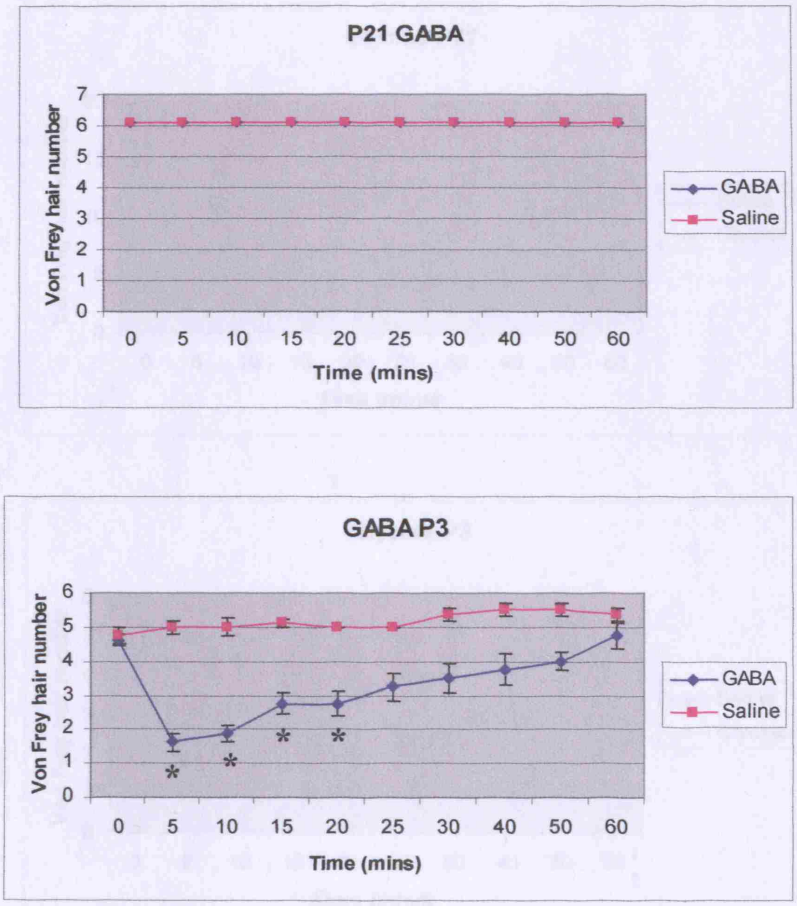
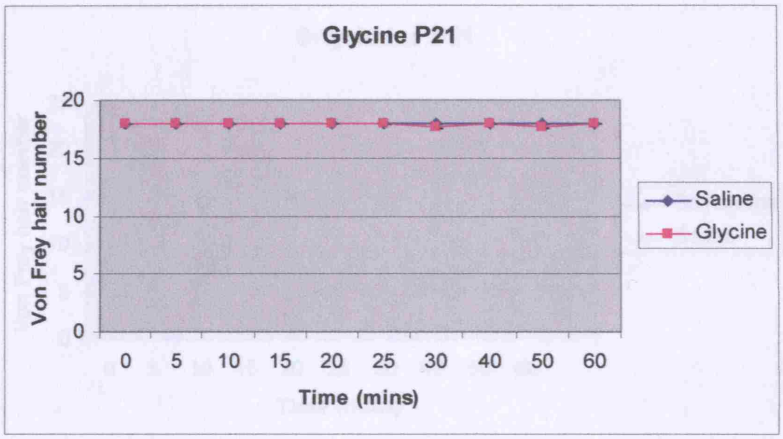


Fig 4.4

Mechanical thresholds following intrathecal administration of GABA and saline vehicle control to rats aged P3 and P21. n = 12 in each group, * P <0.05. P21 animals are unaffected by the application of GABA, whereas P3 pups show a reversible SENSITISATION following IT administration, which remains significant for 20 minutes, with recovery occurring by one hour.

Intrathecal Administration of Glycine



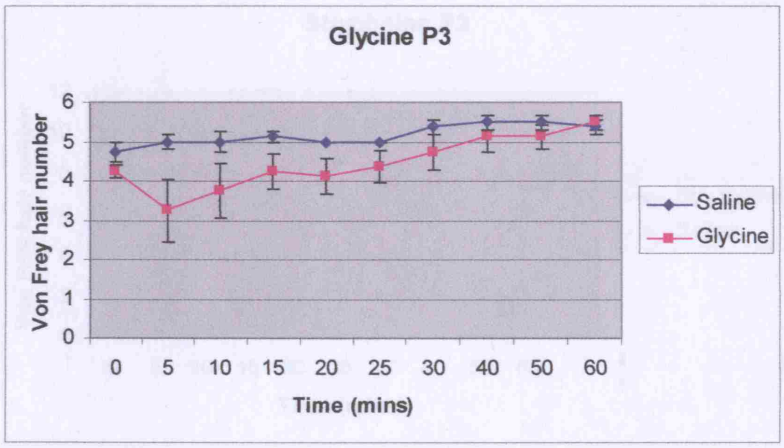


Fig 4.5

Mechanical thresholds following intrathecal administration of glycine and saline vehicle control to rats aged P3 and P21. n = 12 in each group. P21 animals are unaffected by the application of glycine, whereas P3 pups show a reversible SENSITISATION following IT administration,maximal at 5 minutes, with recovery occurring by one hour.

Intrathecal Administration of Strychnine

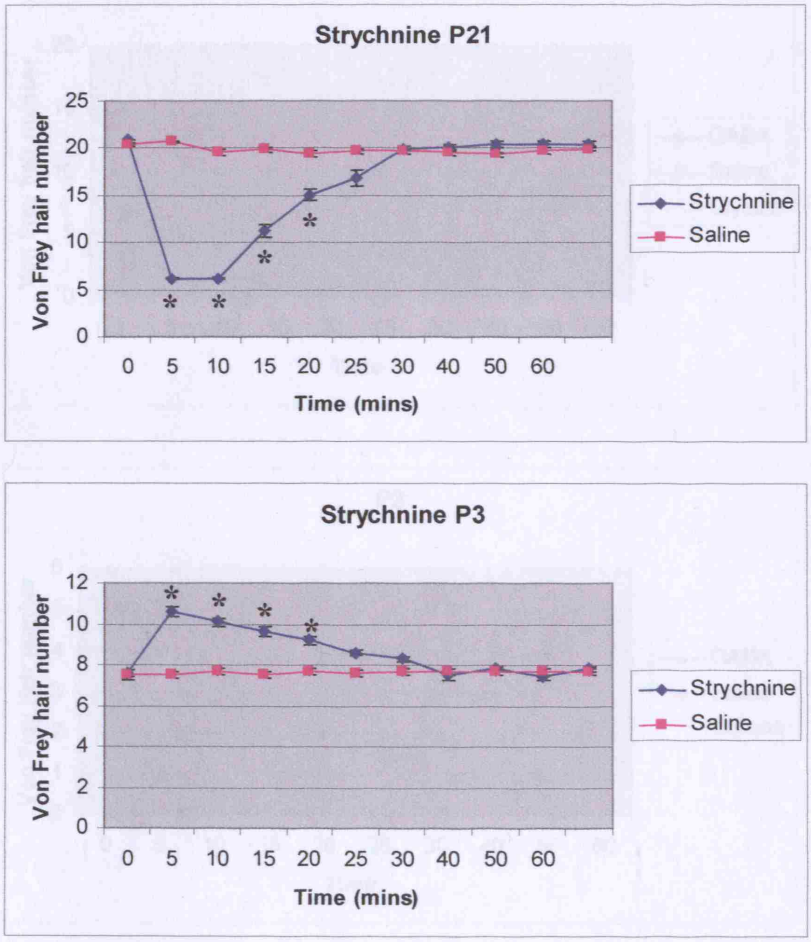


Fig 4.6
Mechanical thresholds following intrathecal administration of strychnine and saline vehicle control to rats aged P3 and P21. n = 12 in each group, * P <0.05. P21 animals are significantly sensitised for 15 minutes following injection, whereas P3 animals show an opposite effect with a RISE in their thresholds for 20 minutes post injection.

Intrathecal administration of GABA & Glycine

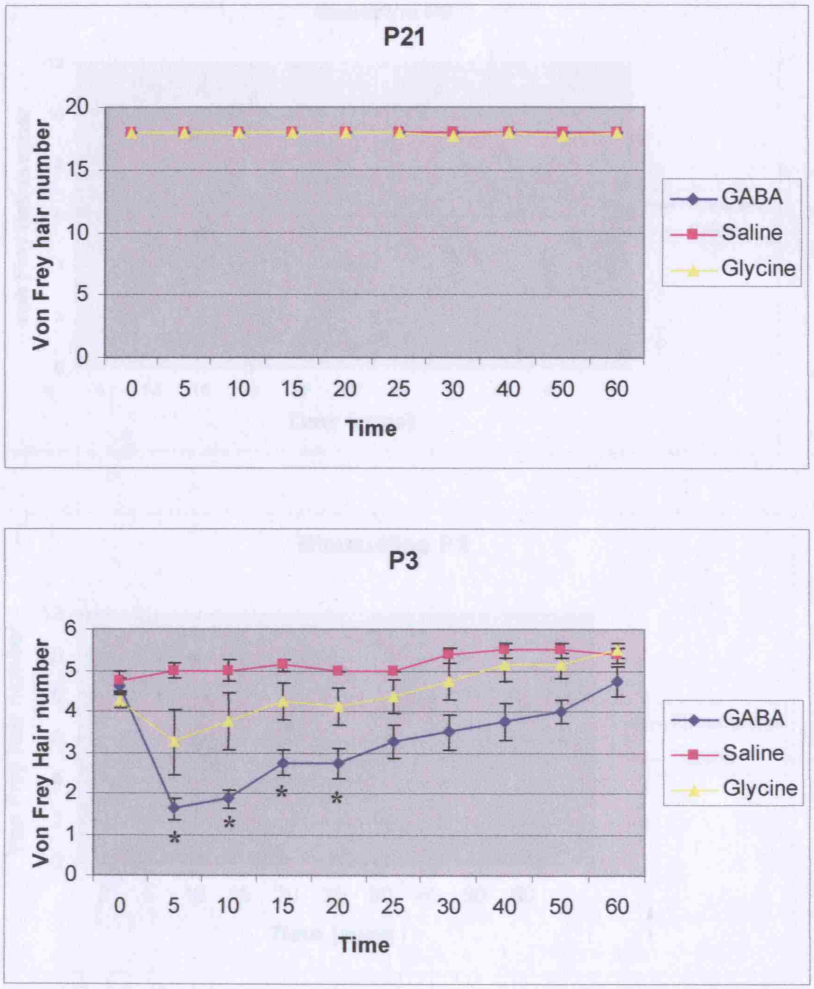


Fig 4.7
Mechanical thresholds following intrathecal administration of GABA, glycine and saline control to rats aged P3 & P21. N=12 in all groups, * P<0.05 (ANOVA). P21 animals are unaffected by the application of GABA & glycine. P3 pups show a reversable sensitisation following IT administration of the compounds, which in the case of GABA is significant for 20 minutes following injection.

Comparison of Intrathecal Gabazine and Bicuculline in P3 rat pups

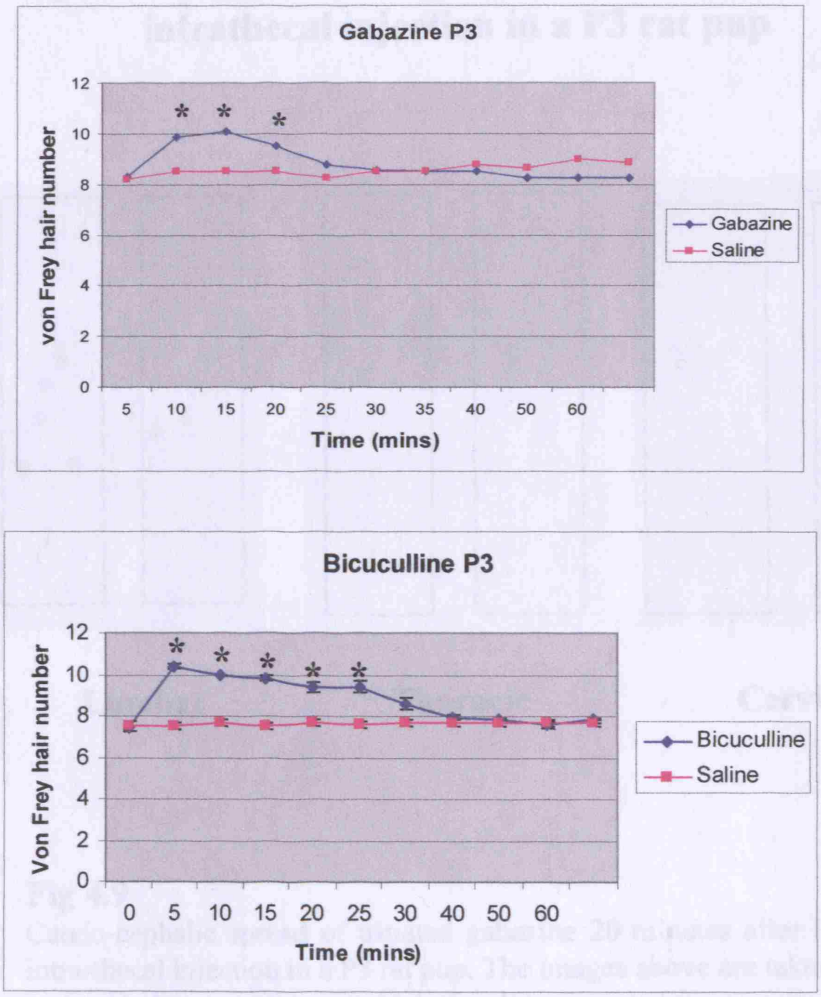


Fig 4.8

Bicuculline was replaced in later experiments by the more selective GABAA antagonist gabazine. Although the effects of both compound are well documented in mature animals, the experiments above were carried out to confirm that our observations in P3 animals following intrathecal bicuculline were reproduced with gabazine (n=12, * $P<0.05$, ANOVA)

Spread of radioactive gabazine following lumbar intrathecal injection in a P3 rat pup

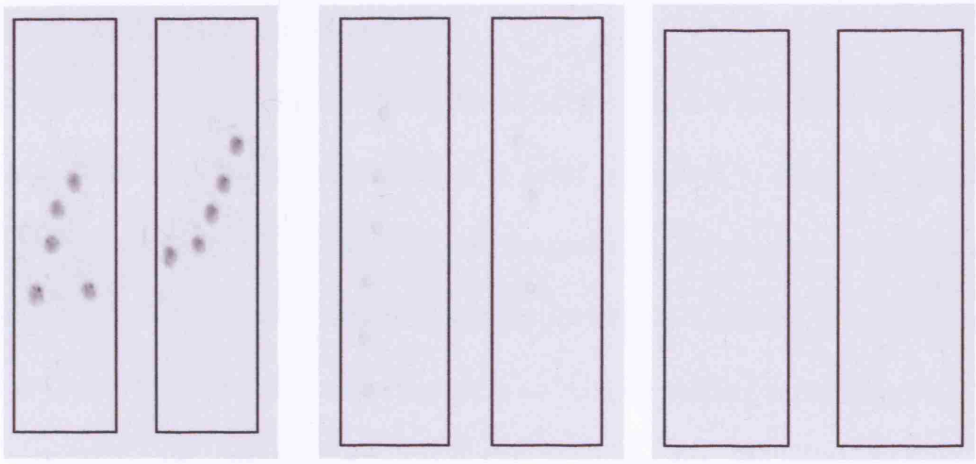


Fig 4.9

Caudo-cephalic spread of tritiated gabazine 20 minutes after lumbar intra-thecal injection in a P3 rat pup. The images above are taken from a phosphoimage screen to which the slide mounted tissuesections had been exposed for 10 days. Gabazine is clearly present in the lumbar sections, with minimal spread to the thoracic region, and only background seen beyond this level.

4.4 Discussion

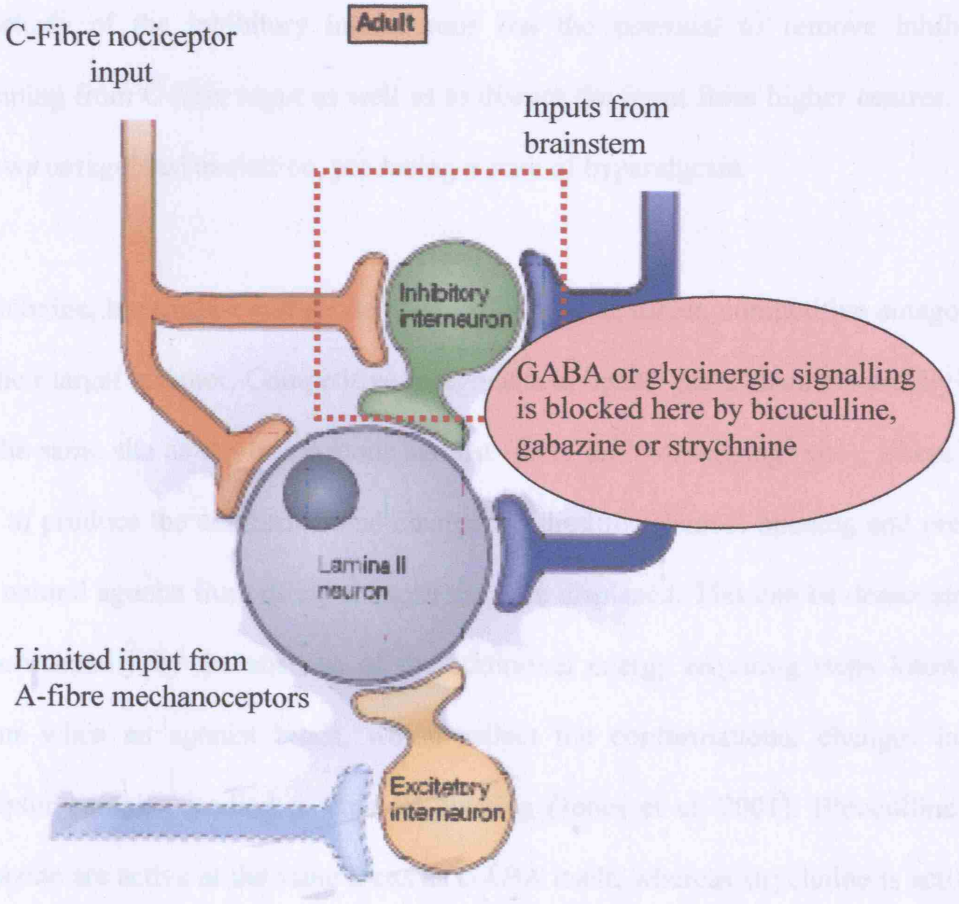
In this chapter, the functional development of GABA and glycine signalling in spinal pain processing over the postnatal period has been examined *in vivo* for the first time. The results show that there is a marked switch in the nature of GABA and glycinergic transmission during the first weeks of life.

4.4.1 Methodological considerations

Mechanical withdrawal thresholds were measured using von Frey hairs applied to the plantar surface of the hindpaw, up to a level sufficient to elicit the cutaneous withdrawal reflex (CWR). This reflex was described by Sherrington in 1910, and has been used as a measure of somatic sensation for almost a century. In adult humans, the nociceptive flexion reflex shows a clear correlation with pain perception in terms of threshold, peak intensity and sensitivity to analgesics (Andrews & Fitzgerald, 1999). It is recognised that in both human infants and rat pups, the flexion reflex can also be evoked with low-intensity mechanical stimuli to the foot, and has a much lower threshold than the nociceptive flexion reflex in the adult (Fitzgerald et al, 1988), such that it may not be a purely nociceptive reflex. This neonatal threshold rises with increasing age (Fitzgerald et al, 1988; Marsh et al, 1999), reflecting a gradual decrease in the excitability of the spinal cord and a reorganisation of connections (Jennings & Fitzgerald, 1996). Correspondingly, the data presented in this study shows cutaneous withdrawal thresholds to be consistent within an age group and to rise with increasing age in accordance with previous findings. The high nature of the mechanical withdrawal threshold in adult rats made it impossible to measure any further increase in threshold, as the von Frey hair producing sufficient force to lift the animal's paw failed to produce a withdrawal response.

Intrathecal injections were carried out according to a validated method devised in adult mice (Hylden and Wilcox, 1980), with a few modifications. Appropriate volumes of injection for the various ages of rat pups used were devised by using aliquots of Evan's blue dye and assessing the apparent rostral spread, by post mortem laminectomy. In order to robustly validate the technique for use in P3 rat pups, radiolabelled gabazine was injected, further demonstrating a lack of rostral spread, and allowing the conclusion that agents injected were acting at the level of the lumbar dorsal horn. So as to allow rapid recovery and early testing, inhalational anaesthesia with halothane was used during the injections. Although, there is some evidence that inhalational anaesthetics depress the response of the spinal dorsal horn low threshold neurons to peripheral receptive field stimulation (Yamauchi et al, 2002); the responses of control (saline injected) animals in this study had returned to baseline by 5 minutes in most cases, and 10 minutes in all cases. The mechanism of the depressed response has been shown to be partly but not completely reversed by strychnine and bicuculline (alone or in combination), implying that halothane has part (but not all) of its depressant effect on spinal sensory neurons via GABA and glycine transmission (Yamauchi et al, 2002). Further work has shown that halothane does not attenuate the development of hyper-excitability of superficial dorsal horn neurons (Kawamata et al, 2005); and that the there is rapid recovery of sensory thresholds after the cessation of the volatile anaesthetic (Yamauchi et al, 2002). This suggests that while inhaled halothane provided ethical intra-operative cover, its effects should have a limited impact on the results subsequently obtained, making it a more useful agent than the intra-peritoneal pentobarbital used in the original method of Hylden & Wilcox.

Bicuculline was substituted for the more selective $GABA_A$ antagonist gabazine in later experiments, in the light of new information relating to the effects of bicuculline on voltage gated potassium currents. Patch clamp data from rat pre-optic neurons has shown that bicuculline is able to block fast potassium currents (Druzin et al, 2004). As the electrochemical gradient for chloride is controlled by a potassium-cation co-transporter (KCC2), this confounding variable was excluded by the change of $GABA_A$ agonists. Gabazine was found to reproduce the effects of bicuculline observed in earlier experiments.



Site of action of IT GABA & glycine antagonists in adult rat. Adapted from Fitzgerald, 2005.

4.4.2 GABA and glycine antagonist administration

The allodynia produced by intrathecal administration of $GABA_A$ and glycine antagonists in adult rodents is well documented (Loomis et al, 2001; Sorkin et al, 1996). This can be shown to be dose dependent (Sherman et al, 1995) and reversible over a reproducible time course (Onaka et al, 1996). In keeping with the work of Onaka and colleagues, the sensitising effects of intrathecal strychnine in adult rats seen in the present study were maximal at 5 minutes, with bicuculline producing its nadir at 10 minutes, and recovery seen by 50 minutes in both cases. The diagram above illustrates the site of action of these compounds in the superficial dorsal horn. Blockade of the inhibitory interneurons has the potential to remove inhibitory damping from C-fibre input as well as to disrupt the input from higher centres. This allows unregulated excitation, producing a state of hyperalgesia.

Strychnine, bicuculline and gabazine are all selective, direct, competitive antagonists at their target receptor. Competitive antagonists at ligand-gated channels usually bind to the same site as the endogenous agonist (or to an 'overlapping' site), where they fail to produce the conformational change required for channel opening and prevent the natural agonist from doing so, until they are displaced. This can be demonstrated experimentally by the absence of the additional energy requiring steps known to occur when an agonist binds, which reflect the conformational changes in the receptor complex leading to channel opening (Jones et al, 2001). Bicuculline and gabazine are active at the same locus as GABA itself, whereas strychnine is active at the same receptor complex as glycine but at a separate closely related site. The compounds are rapidly cleared from cerebrospinal fluid (CSF) and metabolised principally by the liver, with only a small amount being excreted unchanged in the

urine. The time course of action found here is consistent with that previously reported, and is likely to be a reflection of their rapid clearance from CSF, followed by competition from the GABA / glycinergic tone in the spinal cord.

The marked increase in sensitivity described above is the opposite to the effect seen in P3 animals, where administration of $GABA_A$ and glycine antagonists increased the mechanical withdrawal thresholds of the pups. As discussed above, naïve withdrawal thresholds for P3 animals are relatively low, due to lack of inhibitory tone. Administration of bicuculline, gabazine or strychnine increased this threshold significantly, bringing it closer to the less sensitive adult situation. The effect was shown to be reproducible and reversible. Related work in our laboratory showed these results to be dose dependent in all cases, and a corresponding effect was also observed on thermal withdrawal latencies (G Hathway, unpublished data).

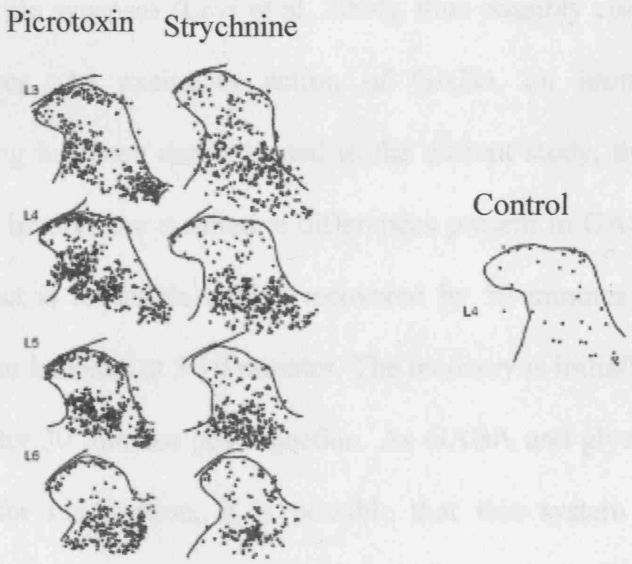
This data demonstrates a previously unknown, developmental 'switch' in the nature of tonic GABA and glycinergic activity *in vivo*. As discussed in chapter 2, changes are occurring in glycinergic and $GABA_A$ signalling in early post-natal life, with an increase in KCC2 and a corresponding reduction in intracellular chloride. KCC2 levels have been shown to be causally linked to the 'switch' in GABA signalling occurring with age (Rivera, 1999), and patch clamp studies have revealed that 40% of superficial dorsal horn neurons produced depolarisation in response to GABA between P0 and P7, although this was not sufficient to produce an action potential (Baccei & Fitzgerald, 2004). The developmental switch has been shown to revert to the neonatal pattern following nerve injury; linking low KCC2 levels with both a reduction in mechanical withdrawal threshold and shorter thermal withdrawal latency

(Coull et al, 2003). It is therefore consistent that we should find opposite reactions to GABA and glycine antagonism in young animals with low levels of KCC2 compared to mature animals with greater expression of the protein.

4.4.3 GABA and glycine administration

Intrathecal $GABA_A$ and glycine administration to adult rats produced no measurable effect. Aside from any measurement difficulties relating to their high threshold, this is likely to be a reflection of the presence of physiological GABA and glycinergic tone in the mature naïve animal, making the addition of further GABA and glycine of little significance. These tonic systems are important in suppressing the development of allodynia and hyperalgesia in the normal animal and their loss may be pivotal in the development of neuropathic pain. Indeed, intrathecal administration of GABA and glycine has been shown to reduce allodynia in experimental pain models (Eaton, 1999; Huang et al, 2000). Huang and colleagues showed that when glycine is administered continuously by intrathecal cannulation, there is a significant reduction in the mechano-nociceptive hyperalgesia produced by sciatic nerve ligation. Interestingly, in their study, Eaton and co-workers showed that in order for intrathecal GABA to reduce the tactile hypersensitivity following nerve injury, it had to be given during a 'window' of 2-3 weeks following the injury, suggesting a role in the induction phase of neuropathic pain. The inhibitory tone in the dorsal horn of adult rats has been anatomically mapped, using the C-Fos response to picrotoxin and strychnine in mature naïve animals. The $GABA_A$ antagonist picrotoxin lead to more overall C-Fos expression, being present in both deep and superficial laminae; whereas strychnine lead to predominantly deep laminae Fos-like immunoreactivity (Cronin et al, 2004). Administration of GABA and glycine to P3 rats lead to a

reproducible, reversible lowering of cutaneous withdrawal thresholds, once again producing the opposite effect to that seen in mature animals. The effects of GABA were of a greater magnitude than those of glycine, which failed to reach statistical significance.



Distribution of C-Fos positive cells in picrotoxin and strychnine treated animals (from Cronin et al, 2004)

Assuming that GABA and glycinergic tone is indeed excitatory during development, it is likely to be under some form of endogenous control. It has been widely suggested that the excitatory actions of GABA and glycine during development serve a trophic role (Ben-Ari, 2002), making precise control of signalling all the more important. Thus adding an external, uncontrolled dose directly on to the spinal cord may indeed be expected to have a significant effect, either locally or in conjunction with higher centres. The greater effect of GABA may be explained by the C-Fos expression pattern shown above, with a more widespread distribution for GABAergic tone including both noxious and non-noxious sensory laminae (Cronin et al, 2004). It

is also interesting to note that unlike GABA, glycine is unable to produce mIPSCs in patch clamped superficial dorsal horn neurons before P10 (Baccei and Fitzgerald, 2004). This may be due to the relative lack of expression of the scaffolding protein gephyrin in the younger neurons, as described in chapter 2. Gephyrin has been shown to be critical for glycine receptor clustering, but not for the formation of functional GABAergic synapses (Levi et al, 2004), thus possibly contributing to any observed differences. An excitatory action of GABA on immature spinal nociceptive processing has been demonstrated in the current study, the mechanism of which is likely to involve the qualitative differences present in GABAergic tone in early life. The effect is reversible having recovered by 50 minutes post injection, and has a maximum intensity at 5-10 minutes. The recovery is initially rapid, appearing to slow down after 30 minutes post-injection. As GABA and glycine rely principally on re-uptake for inactivation, it is possible that this system becomes saturated by a relatively large exogenous dose of neurotransmitter. The principle reuptake transporter for GABA (GAT1) is also know to be dependent on co-transport of Na^+ and Cl^-, and may thus be affected by the neonatal reversal in chloride flux (Lu & Hilgemann, 1999), further accounting for the relatively long duration of action seen.

4.4.4 Conclusions

In this chapter, an excitatory role has been demonstrated for GABA and to a lesser extent glycine in the immature rat, *in vivo*. The effect of bicuculline, gabazine and strychnine on GABA and glycinergic tone is also found to be qualitatively different in P3 pups. These observations *in vivo* are consistent with the developmental findings *in vitro* in chapter 2, and have potentially wide reaching implications for the use of GABAergic drugs in neonatal patients (see appendix 1).

Chapter Five

Preliminary Investigations of the Contribution of Higher Centres to the Developmental 'Switch' of GABA Signalling *in vivo*

5.1 Introduction

In Chapter 4, we have demonstrated clear developmental differences in the nature of GABA and glycinergic signalling in animal behavioural studies. The effect of intrathecal injection of GABA, glycine and their antagonists was found to be opposite in P3 rat pups to that observed in mature animals. Although the effect found was significant, reversible and reproducible, the complicated nature of sensory processing at the level of the spinal cord means that further work is required to confirm the exact site and mode of action. Although, Patch clamp recordings from single superficial dorsal horn neurons at P0-P7 showed depolarisation to GABA in 40% of cases this was not sufficient to produce action potentials (Baccei & Fitzgerald, 2004), and application of gabazine to these neurons was found to be predominantly excitatory (ML Baccei, unpublished data). Despite, radiolabelled gabazine studies confirming a local site of action for the compounds injected, an isolated superficial dorsal horn mechanism therefore appears unlikely. In order to reconcile these differences between single cell electrophysiological data, and behavioural observations we sought to investigate the importance of descending connections.

The principle benefit of conducting the studies *in vivo* is that the spinal cord is being examined in the presence of its normal anatomical connections to the higher centres involved in pain processing. Ascending projections from dorsal horn neurons have been shown to be present from two thirds of the way through gestation in the foetal sheep (Rees et al, 1994), implying that ascending pathways to the brainstem and cortex are probably mature at birth. Descending projections from the brainstem to the superficial dorsal horn of the spinal cord are known to be important in pain

modulation. As with ascending tracts, it appears that axons from brainstem nuclei grow down the spinal cord well before birth. Injections of horseradish peroxidase into the lumbosacral spinal cord of the neonatal rat label brains stem nuclei with similar density to that seen in the adult (Leong, 1983). Functional studies have failed to produce descending inhibition as a result of dorsolateral funiculus stimulation before the age of P9 (Fitzgerald & Koltzenburg, 1986) suggesting a lack of descending inhibition. Interestingly, these studies did not attempt to measure the presence of descending excitation, which is now known to exist in mature animals. The rostroventral medulla (RVM) at the level of the facial nucleus (nucleus raphe magnus, and reticularis gigantocellularis pars alpha) is known to play an important part in the modulation of nociception (see fig 5.1). Although there is no obvious pattern of topographical organisation of the RVM nuclei, there is evidence for distinct classes of neurons based on their functional characteristics (Suzuki et al, 2004). The two main types of cells are known as 'ON' cells (inhibited by opioids and stimulated by nociceptive input) and 'OFF' cells (indirectly stimulated by opioids and inhibited by nociceptive input). The balance between these cells ultimately determines the output of the RVM, which is relayed to the dorsal horn via serotonergic neurons (Suzuki et al, 2004). The RVM is also known to contain GABAergic interneurons (Gilbert et al, 2001), and little is known at present about the development of these cells.

In order to differentiate effects produced solely on a local, spinal cord, level from those involving this descending input, another set of experiments were carried out in spinalised animals, in which such connections are absent. The presence of some descending input as early as P3 was investigated by unilateral spinal cord injection of

the retrograde tracer fluorogold into rats of different ages followed by further developmental immunohistochemical studies of KCC2 expression in the region of the RVM. Thus, in this chapter, an attempt is made to elucidate the anatomical mechanism of the developmental 'switch' in the behavioural response to GABA described earlier in this thesis.

5.2 Materials and Methods

Sprague Dawley rats of both sexes were used. These were bred by the Central Animal Facility, University College London, and were kept in artificial lighting on a 12:12h light cycle with the temperature maintained at a constant 21°C. Food and water were given *ad libitum* and all procedures were carried out in accordance with the United Kingdom Animal Procedure Act 1986. P3 and P10 rat pups were housed with their mother and littermates. P21 animals were housed independently in groups of 6. All behavioural experiments were carried out with a specific set of Von Frey hairs, which were calibrated prior to the study.

5.2.1 Spinal Cord Transection

Spinal cord transection was performed in a group of eight P3 rat pups. These pups were anaesthetised with halothane (2-4%) in oxygen, a laminectomy was performed in the upper thoracic region and the cord was divided. Animals were allowed to recover for 2-3 hours prior to intrathecal injections (as described in Chapter 4) and behavioural investigations were carried out as before. At the end of the experiment, the upper thoracic cord was dissected, and data only included if complete transection was confirmed. This work was carried out in conjunction with Dr Gareth Hathway and Dr Suellen Walker. Dr Walker carried out the spinalisations, and Dr Hathway and myself conducted the behavioural testing.

5.2.2 Fluorogold Injections

Rats aged P3 and P21 were lightly anaesthetised with 4% halothane in O_2 in a gas box and maintained at 1.5% halothane via a mask. The animals were stabilised in a

metal frame at the level of the ears and pelvis. A laminectomy was then performed at the level of the lower lumbar region, and 400 ηl of the retrograde tracer fluorogold was injected in to the left side of the spinal cord at the level of L4-L5 (lumbar enlargement) to a depth of 640 μm (based on the methods of Koltzenburg & Fitzgerald, 1986) in P21 animals and 350 μm in P3 pups. The animals were then recovered and given post surgical analgesia with buprenorphine 0.5mg/kg i.m. (Alstoe Animal Health). P3 pups were sacrificed after 16 hours and P21 rats after 3 days; in order to allow adequate time for the fluorogold to be transported. This work was carried out in conjunction with Sharon Man, who conducted the surgery with me jointly.

5.2.3 Immunohistochemical Studies of the Brainstem during Development

After the fluorogold injections the rats, aged P3 and P21, were terminally anaesthetised as above. They were then perfused trans-cardially first with heparinised saline, followed by a fixative solution containing 4% paraformalderhyde in 0.1M phosphate buffer. The brainstems were removed, and post-fixed in the same solution for 2 hours at room temperature. The brainstem segments were then cryo-protected by overnight immersion in a 30% sucrose phosphate buffer at 6°C. Following this they were mounted in Tissue-Tek OCT compound (Sakura Finetek) and transverse sections of 20 microns were cut, using a Leika cryostat, through the entire brainstem region. These were thaw-mounted on to gelatinised slides, and then the sections were blocked in a solution of 0.1M PBS, containing 5% normal goat serum (Vector), and 0.25% fish gelatin (Sigma), for 2 hours at room temperature. Next they were incubated in primary antibodies, made in 0.05M tris-saline with 0.3% triton X-100 (TTBS), and 5% normal goat serum, overnight at 6°C.

These consisted of:

- rabbit polyclonal antibody raised against KCC2 (Chemicon) at 1:2000, used in conjunction with tyramide amplification, as previously described (section 2.2.1);
- rabbit polyclonal anti-fluorogold (Flurochrome, USA) at 1:200 with biotin amplification; and
- mouse monoclonal anti-NeuN (Chemicon) at 1:500 also used with biotin amplification.

As usual, control sections were treated in the same way except for omission of the primary antibody. Following the final washes sections were cover slipped.

5.3 Results

In this chapter, further behavioural experiments reveal the importance of intact supraspinal connections to the developmental 'switch' observed previously in the behavioural response to intrathecal gabazine. Preliminary immunohistochemical experiments are carried out in order to further elucidate the nature of these connections.

5.3.1 The postnatal switch in response to intrathecal gabazine was not observed in spinalised animals (see fig 5.2)

In order to test the effect of descending inhibition / facilitation on the behavioural response to intrathecal gabazine, the drug was administered to spinalised P3 rats, which were therefore lacking input from higher centres. Under these conditions, the response to gabazine reverted to the pattern seen in the mature animal, and significant, reversible lowering of mechanical thresholds occurred ($n=8$, $P<0.05$, ANOVA). This strongly implies that the post-natal switch in GABAergic control of spinal nociception is supraspinally mediated.

5.3.2 Injection of the retrograde tracer fluorogold in to the lumbar spinal cord labels cells in the region of the rostro-ventral medulla at both P21 and P3 (see fig 5.3 & 5.4)

The retrograde tracer fluorogold was injected into the left side of the spinal cord of rats aged P3 and P21 ($n=4$ animals at each age, 3 section were examined from each). Staining was found bilaterally in the area of the rostro-ventral medulla (RVM) in both ages. This would seem to imply that the descending pathways via the RVM are mature in P3 rats, and as such may be important in the mechanism of the behavioural

'switch', as the RVM is known to be a particularly important site for integrating descending influences to the spinal cord (Suzuki et al, 2004).

5.3.3 Expression of KCC2 in the region of the rostro-ventral medulla is postnatally regulated (see figs 5.3, 5.4 & 5.5)

Sections of brainstem from rats aged P3 and P21 (n=4 animals at each age, 3 sections examined from each) were labelled immunohistochemically for the cation-chloride cotransporter KCC2, which is known to undergo developmental upregulation in many areas of the CNS (Ludwig et al, 2003; Leitch et al, 2005; Stein et al, 2004). The neuronal nuclear marker NeuN was used as a control for nuclear density. The amount of KCC2 labelling relative to NeuN staining was found to be considerably less at P3. This suggests a postnatal up-regulation of KCC2. This in turn could imply an excitatory role for GABA in the RVM, which is known to have significant GABAergic transmission.

5.3.4 Following lumbar spinal injection of fluorogold, the labelled cells co-localise with KCC2 in the rostro-ventral medulla (see fig 5.6)

Finally, the cells labelled with fluorogold, following lumbar spinal injection of the tracer were double-labelled with KCC2 antibody. The labelled cells colocalised with KCC2 at both ages. However, the relative proportion of KCC2 per labelled cell was found to be much greater in the older animals.

Orientation of the Rostroventral Medulla Within the Rat Brainstem

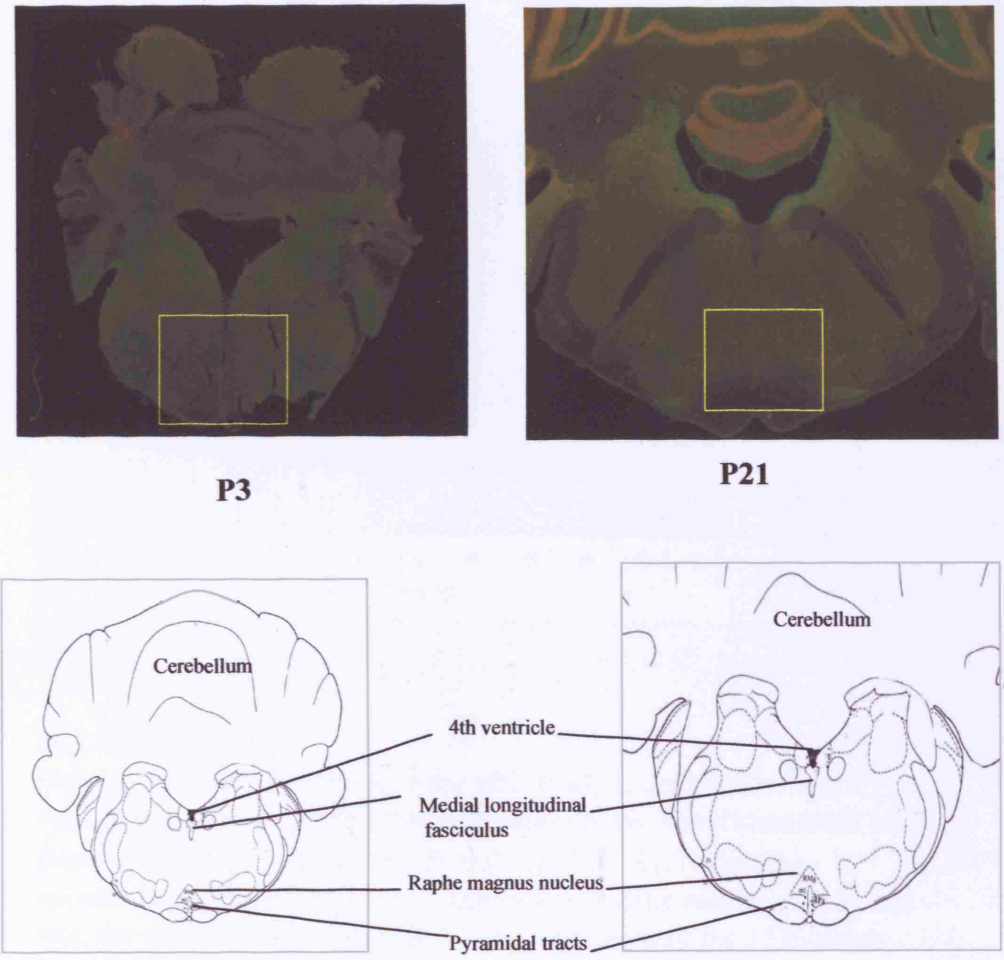


Fig 5.1
Diagrams illustrating the anatomical landmarks in the region of the RVM

Comparison of the Effects of Gabazine in Spinalised and Intact P3 Rat Pups

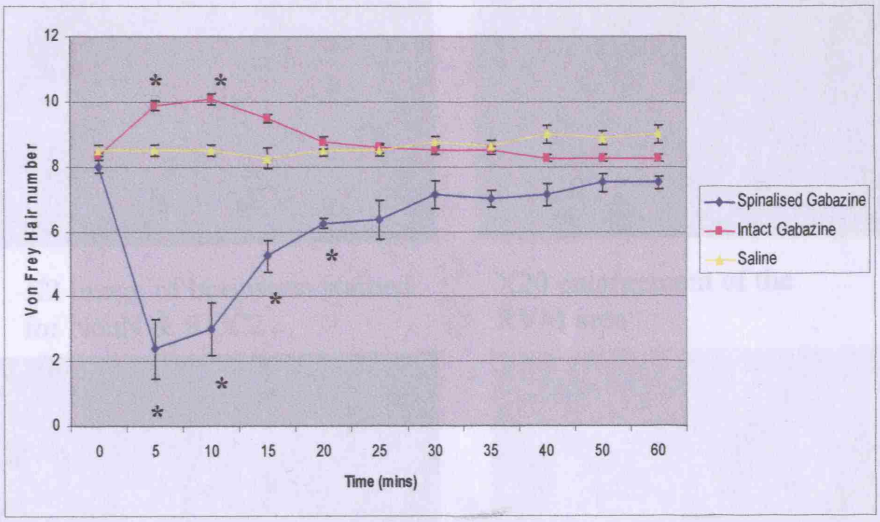
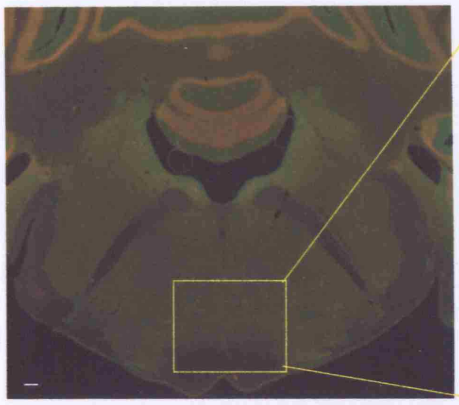


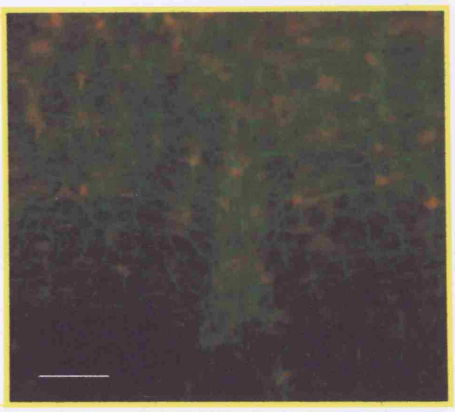
Fig 5.2

Gabazine is seen to reproduce the effects of bicucculine in intact young rats, with reversible desensitisation being significant until 15 minutes post injection n = 8, *P<0.05 (ANOVA). Following spinalisation, the effect is more reminiscent of the results seen in P21 rats, the mechanical thresholds **falling** significantly for 25 minutes before recovering by 50 minutes post administration; n = 6, *P<0.05.

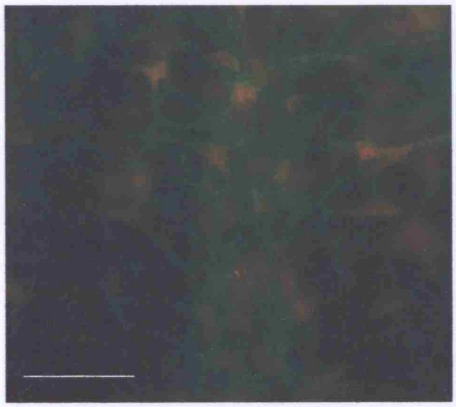
P21 Rat Brainstem at the Level of the Rostro-ventral Medulla



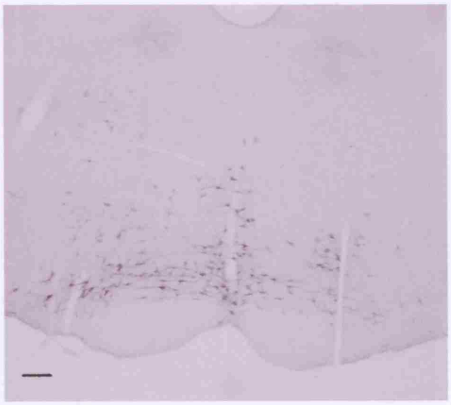
X2 image of brainstem stained for NeuN & KCC2



X20 enlargement of the RVM area



X40 image of RVM

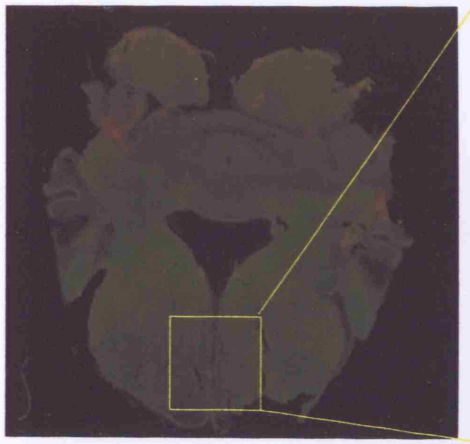


X4 image of brainstem following fluoro-gold injection to left lumbar region. Fluoro-gold positive cells are stained black with DAB.

Fig 5.3

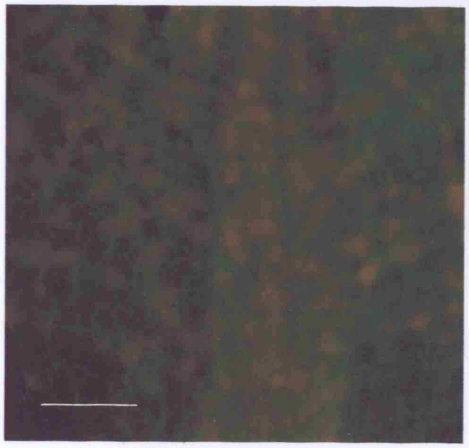
The expression of KCC2 (green stain) in this region of the brainstem is shown along side a neuronal nuclear marker (red stain). The fluoro-gold injection demonstrates the regions of the brainstem sending input to the dorsal horn (shown in black). Scale bars 20 μm.

P3 brainstem at the Level of the Rostro-ventral Medulla

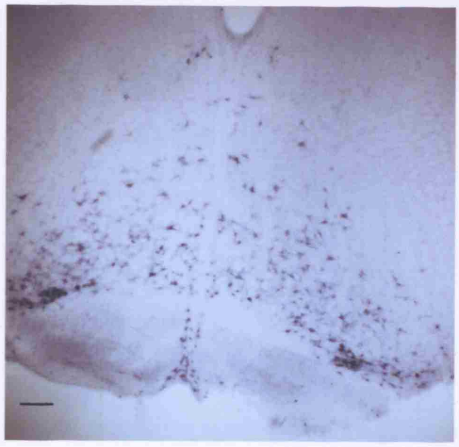


X2 image of brainstem stained for NeuN & KCC2

X20 enlargement of RVM area



X40 image of RVM

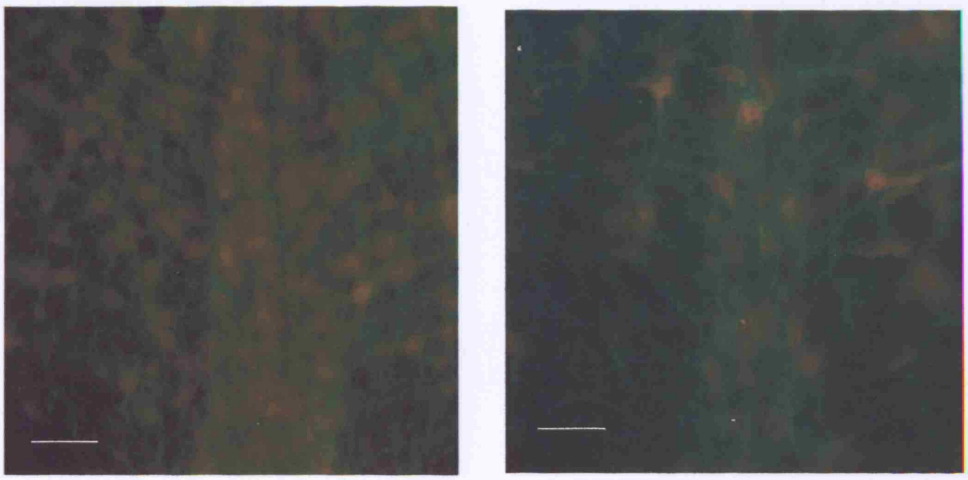


X4 image of brainstem following fluoro-gold injection to left lumbar region. Fluoro-gold possitive cells are stained black with DAB.

Fig 5.4

The expression of KCC2 (green stain) in this region of the brainstem is shown along side a neuronal nuclear marker (red stain). The fluoro-gold injection demonstrates the regions of the brainstem sending input to the dorsal horn (shown in black). Scale bars 20 μm.

Direct Comparison of KCC2 Expression Relative to Neuronal Density at P3 and P21



P3 RVM X40

P21 RVM X40

Fig 5.5

Comparison of P3 and P21 RVM, demonstrating the relatively greater expression of KCC2 per neuronal nucleus at P21 NeuN (red) shows the neuronal nuclei, and KCC2 is stained green. Scale bars 20μm.

Confocal Images of the Brainstem at the Level of Rostro-ventral Medulla Stained for KCC2 & Fluorogold

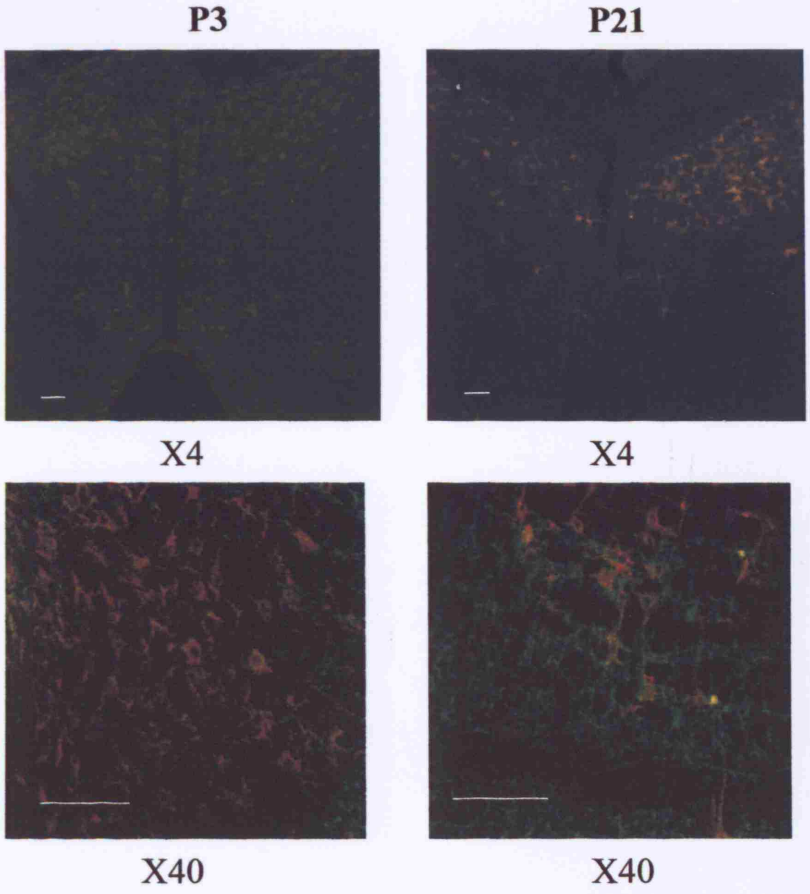
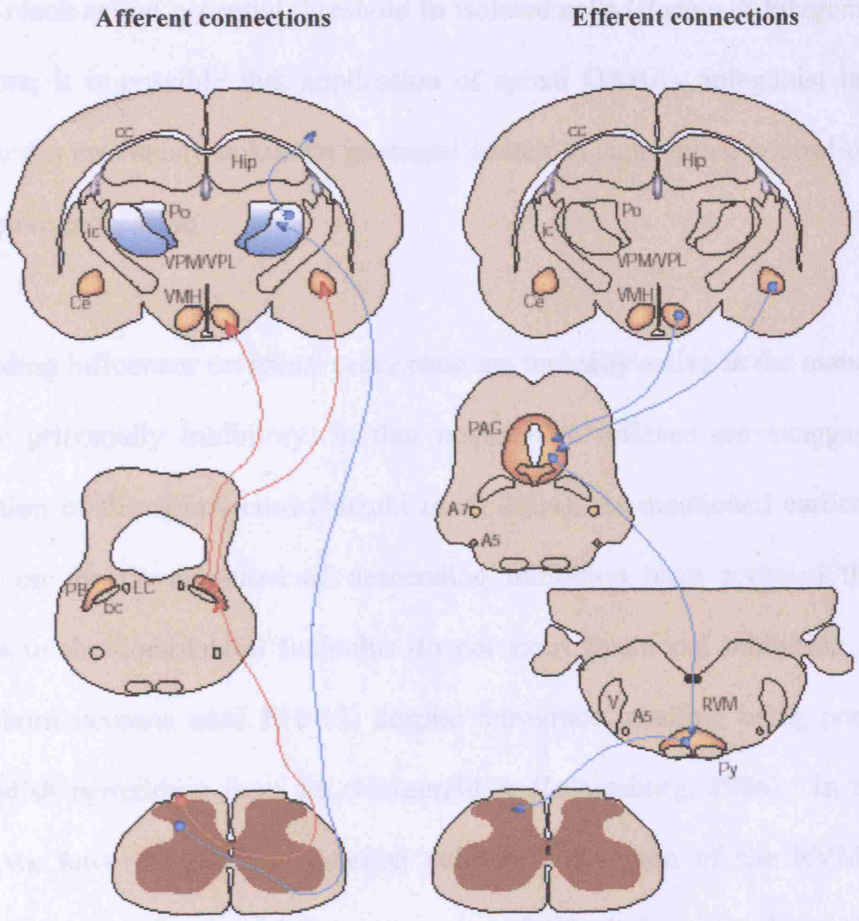


Fig 5.6
Co-localisation of KCC2 reen (green stain) on RVM neurones stained with fluorogold (red stain) following lumbar spinal injection of the tracer. The mature brainstem sections appear to have a greater expression of KCC2 per projection neurones than the P3 tissue. Scale bars50 μm.

5.4 Discussion

The advantage of conducting these experiments *in vivo* is that it allows the maturational processes in the spinal cord to be evaluated in the presence of normal anatomical connections, many of which are also undergoing developmental changes. Manipulation of these connections by spinalisation, has revealed the importance of descending input in the behavioural response to intrathecal gabazine.



Dorsal horn connections with higher centres, Hunt & Mantyh, 2001

(PAG-Periaqueductal grey matter, RVM-Rostroventral medulla, Hip-Hippocampus, CC-corpus callosum, Ce-central nucleus of amygdala, ic – internal capsule, Py- pyramids, PB – parabrachial area, V – ventricle, VMH, VPL, VPM – nuclei of hypothalamus)

Indeed, the postnatal 'switch' in the effect of gabazine was not observed in spinalised P3 rat pups, whose responses resembled those of the mature P21 animals. Thus, GABA appears to exert an early postnatal excitatory control, only observed when supraspinal connections are intact, which later switches to inhibitory control. A supraspinal contribution to the effect would be in keeping with patch-clamp data from neonatal superficial dorsal horn neurons, in which GABA is depolarising, but fails to reach action potential threshold in isolated cells (Baccei & Fitzgerald, 2004). Therefore, it is possible that application of spinal $GABA_A$ antagonist in vivo has unmasked a previously unknown postnatal switch in supraspinal control over spinal nociceptive processing.

Descending influences on spinal processing are tonically active in the mature animal, and are principally inhibitory, in that nociceptive reflexes are exaggerated after transection of the spinal cord (Suzuki et al, 2004). As mentioned earlier, previous studies on the development of descending inhibition have revealed that certain neurons in the dorsolateral funiculus do not exert functional inhibition on lumbar dorsal horn neurons until P10-12, despite retrograde labelling being possible with horseradish peroxidase from P6 (Fitzgerald & Koltzenburg, 1986). In the present study, we have successfully labelled cells in the region of the RVM with the retrograde tracer fluorogold from the age of P3. It has also been shown that spinalised P3 animals behave in a qualitatively different manner when gabazine is administered intrathecally. This provides evidence for earlier existence of descending communications between the brainstem and the lumbar dorsal horn than was previously documented. Immuno-labelling of KCC2 in the region of the RVM showed an apparent postnatal upregulation of the transporter, when changes in

neuronal density are controlled for by double labelling with the neuronal nuclear marker NeuN. Double labelling of RVM neurons with fluorogold and KCC2 revealed colocalisation, suggesting that at least some of the neurons modulating descending inhibition express the transporter molecule. Developmental changes in KCC2 could therefore have an important impact on the nature of descending signals. Dorsal horn ascending pathways activate both inhibitory and facilitatory descending controls from the RVM (Suzuki et al, 2004), through activation of 'ON' and 'OFF' cells, which mediate excitatory and inhibitory effects on spinal nociceptive processing (Neubert, 2004; Suzuki et al 2004). It is conceivable that the neonatal balance of these brainstem facilitatory and inhibitory controls may differ from the adult situation, perhaps due to immature GABAergic transmission in 'ON' and 'OFF' cells. The role of GABAergic signalling in dictating the output from the RVM has been demonstrated by local injections of muscimol and bicuculline, which produced opposite effects on noxious processing (Gilbert et al, 2001). GABA is also the main inhibitory neurotransmitter in the periaqueductal grey matter (PAG), where developmental changes have been shown to occur in the amplitude of the mIPSCs generated (Hahm et al, 2005). Differential developmental upregulation of KCC2 within different cell populations of a particular region in the CNS has been demonstrated in the mitral cells and granule cells of the rat olfactory bulb. Wang and colleagues showed that although mitral cells had KCC2 mRNA levels approaching adult values from the time of birth, granule cells had undetectable levels for the first week, and approached mature levels only after P14 (Wang et al, 2005). They went on to examine the response of the two cell type to GABA using patch clamp recordings, and found the granule cells to display depolarisation in response to GABA for the first 2 weeks of life, in accordance with their low level of KCC2, whereas mitral cells

had a hyperpolarizing response throughout. A similar differential upregulation of KCC2 in the RVM, between 'ON' cells and 'OFF' cells could account for the differences in behavioural response observed.

Thus, the mechanism involved in the developmental behavioural 'switch' in GABA signalling is not simply a local effect at the level of the dorsal horn, but rather is dependent on intact supra-spinal connections. Retrograde labelling has revealed anatomical projections from the dorsal horn to the RVM present from P3 onwards, which colocalise with KCC2. The expression of KCC2 is shown to be greater in older animals, and the effect of this on GABAergic signalling may in turn be affecting the balance between the output of 'ON' and 'OFF' cells.

Chapter 6

Conclusions

6.1 Introduction

The work described in this thesis provides insights into the development of inhibitory synapses in spinal pain pathways both on the molecular level, and from behavioural studies. The practical relevance of these early postnatal changes to clinical practice is revealed in appendix 1. Although the results presented have been extensively discussed within each chapter, the aim of this section is to summarise our findings and provide a broader overview of their significance.

6.2 Summary of findings

Studies of spinal pain processing in both human infants and rat pups have long established a relative lack of inhibitory control (Fitzgerald, 1999). This thesis has therefore sought to investigate the molecular basis for these observations by studying the postnatal development of inhibitory synapses. GABA and glycine are the principle inhibitory neurotransmitters at the level of the spinal cord dorsal horn, and this work has therefore been focused on developmental changes in inhibitory amino acid signalling. We have demonstrated postnatal changes in the expression of two functionally important proteins located at inhibitory synapses. The first of these was gephyrin, which is known to be a scaffolding protein important in the maintenance of postsynaptic receptor clusters at symmetrical synapses (Sassoe-Pognetto & Fritschy, 2000). This structural molecule is essential for normal glycinergic signalling (Kirsch et al, 1993), and also has a role in the clustering of $GABA_A$ receptors (Essrich et al, 1998). Using immunohistochemistry and western blot analysis, we have shown a postnatal upregulation of gephyrin at the level of the dorsal horn occurring between P10 and P21. The protein expression increased in a ventrodorsal fashion analogous to the earlier development of GABAergic terminals (Alain et al, 2004). Despite the

presence of functional glycine receptors, patch clamp recordings from superficial dorsal horn neurons before the age of P10 shown a relative absence of miniature inhibitory postsynaptic currents in response to glycine application (Baccei & Fitzgerald, 2004). It is likely that the comparatively late appearance of gephyrin at the postsynaptic membrane may be responsible. In the wider context, a relative lack of inhibitory receptor clustering could greatly reduce the effectiveness of inhibitory interneurons, both segmentally in the dorsal horn itself, as well as in higher centres important in the processing of descending inhibition. Thus the postnatal upregulation of gephyrin in the dorsal horn, shown here for the first time, may play an important quantitative role in the observed differences in neonatal pain processing. Previous studies have suggested that gephyrin upregulation in other regions of the CNS is reliant on the presence of intact neuronal connection (Eleore et al, 2005), but is independent of reductions in neuronal activity (Seitanidou et al, 1992). In our study, the developmental changes in gephyrin expression were unaltered by the presence of peripheral inflammation suggesting that neither an increase in primary afferent input, nor a reduction in neuronal activity (as previously described) affect the upregulation of gephyrin, in the intact animal.

The second protein examined was the neuron specific cation co-transporter KCC2. This molecule is important for the active extrusion of chloride ions from neurons, needed to maintain the mature electrochemical gradient required for the inward movement of chloride ions during receptor channel opening. Absence of KCC2 therefore causes increased intracellular chloride and reversed chloride flux leading to depolarisation rather than hyperpolarisation in response to GABA or glycine signalling (Rivera, 1999). KCC2 is known to undergo postnatal upregulation in

several areas of the CNS, (Clayton et al, 1998; Lu et al, 1999). An earlier study of the developmental expression of KCC2 in the rat spinal cord suggested that no change occurred beyond the stage of P3 (Stein et al, 2004), however in this case no attempt was made to separate the ventral and dorsal horns. Our imunohistochemical experiments did indeed show strong KCC2-like immunofluorescence in the motoneurons from P3 onwards, but the expression in the dorsal horns appeared quite different. Very little KCC2 was seen in the dorsal horns before the age of P10, and the dense band of expression in the superficial laminae present in mature animals was not seen until P21. Western blot analysis of the isolated dorsal horns revealed a significant postnatal upregulation occurring between P3 and P10. Reduction in KCC2 in the spinal dorsal horn of rats following nerve injury has been shown to reduce mechanical thresholds and thermal withdrawal latencies (Coull et al, 2003), and the low levels of KCC2 found before P10 could similarly have a qualitative effect on GABA and glycinergic signalling in the neonatal spinal cord. In the case of KCC2, peripheral inflammation caused a significant acceleration in expression suggesting that the process of upregulation may be activity dependent.

This last observation was further investigated using isolated dorsal horn neurons grown *in vitro*, thus allowing more specific manipulation of the extracellular milieu during early development. KCC2 upregulation was found to be reduced by the presence of the sodium channel blocker TTX and accelerated by the addition of supra-physiological concentrations of potassium to the medium, confirming an element of activity dependence. However, addition of a 'receptor inhibitor' cocktail containing antagonists for GABA, glycine and glutamate receptors failed to produce an effect. Patch clamp recordings from neonatal superficial dorsal horn neurons have

shown them to posses spontaneous activity (Baccei & Fitzgerald, 2005), and it appears likely that this is the source of the upregulatory drive. In support of this argument, KCC2 upregulation begins in culture at a stage that predates the formation of synaptic connections.

Thus, we may postulate that postnatal gephyrin upregulation may be responsible for quantitative differences in synaptic inhibition, and that low neonatal levels of KCC2 could actually produce a qualitatively different, excitatory output following the activation of $GABA_A$ or glycine receptors. These hypotheses were tested by intrathecally administering GABA, glycine and their antagonists to rat pups at different stages of development, before testing their mechanical withdrawal thresholds. The effects of administering GABA and glycine antagonists intrathecally to mature rodents are well known (Loomis et al, 2001; Sorkin et al, 1996) producing a lowering of mechanical thresholds that has been used as a model for the investigation of chronic pain states. We were indeed able to reproduce these findings in P21 animals. Interestingly, P3 rat pups exhibited the opposite behavioural consequence to mature animals following the administration of gabazine and strychnine, with their classically low neonatal thresholds actually increasing (becoming LESS sensitive). Similarly, GABA and glycine, which produced no measurable effect in P21 animals, caused a reversible lowering (sensitisation) of mechanical withdrawal thresholds at P3. These results are consistent with our observations relating to the development of inhibitory synapses.

Patch clamp recordings from superficial dorsal horn neurons prior to P6 reveal a depolarising action of GABA in 40% of cases, however this does not meet the

threshold for action potential (Baccei & Fitzgerald, 2004). This argues against a purely local mechanism at the level of the dorsal horn for the behavioural observations described above. Experiments conducted with radiolabelled gabazine confirmed that the site of action of the drugs themselves was indeed at the level of the lumbar spinal cord, as minimal spread occurred rostrally. However, when the study was repeated with spinalised P3 animals, in which there was no supra-spinal input, the response to gabazine reverted to that of the mature animal, with sensitisation of mechanical withdrawal thresholds. The excitatory response to intrathecal GABA demonstrated in P3 rat pups is therefore shown to be dependent on intact supra-spinal connections.

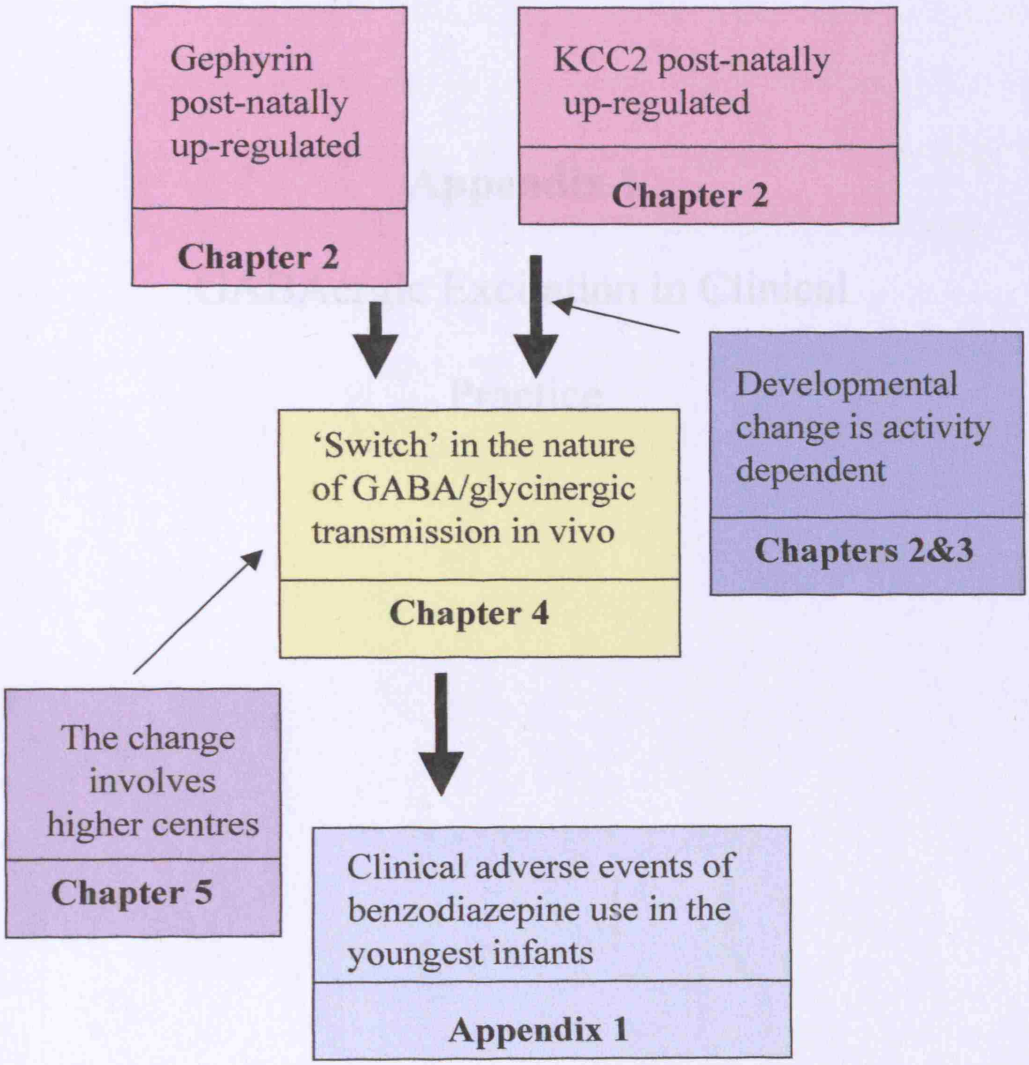
Retrograde labelling was used to demonstrate anatomical projections from the dorsal horn to an area of the brainstem corresponding with the rostroventral medulla, which were present from P3 onwards. These projections were shown to co-localise with KCC2 expression in the RVM. Developmental immunohistochemical studies of KCC2 in the RVM revealed a greater expression at P21 compared to P3. It is therefore possible that the resulting differences in GABAergic signalling could have an effect on the nature of the descending modulation of pain accounting for our behavioural observations.

Many preterm infants on neonatal intensive care units receive benzodiazepines as sedation for procedures or during mechanical ventilation. Benzodiazepines are effective via a modulatory site on the $GABA_A$ receptor that increases chloride flux for a given amount of GABA release. It therefore follows from our observations in the laboratory that the response of the youngest infants to these drugs may be

different from that of an adult patient. Interestingly there are a number of case reports in the medical literature relating to paradoxical neurological adverse events of benzodiazepines in neonates (Waisman et al, 1999; Chess et al, 1998; Zaw et al, 2002; Ng et al, 2002). These consist principally of seizures and myoclonic movements, and in at least one case were shown to be reversed by the benzodiazepine antagonist flumazenil (Zaw et al, 2001). In order to assess the magnitude of the problem in neonatal care in the UK, a postal questionnaire was devised, which asked clinicians to report on their experience of all adverse events experienced with benzodiazepine use. The clinicians responding reported a significant rates of seizures and myoclonic movements, and these were over represented amongst the youngest (<32 weeks PCA) infants in accordance with our findings in the laboratory.

6.3 Concluding remarks

This thesis has therefore contributed to an understanding of the molecular developmental changes occurring at inhibitory synapses. The mechanism of these changes has been investigated, and the relationship to neuronal activity assessed. The behavioural impact of differences in GABA and glycine signalling has been demonstrated *in vivo* for the first time, and contributions of supra-spinal connections have been examined. Finally, the clinical impact of these maturational differences has been assessed, and while these observations alone are insufficient to change clinical practice, they pave the way for further research.

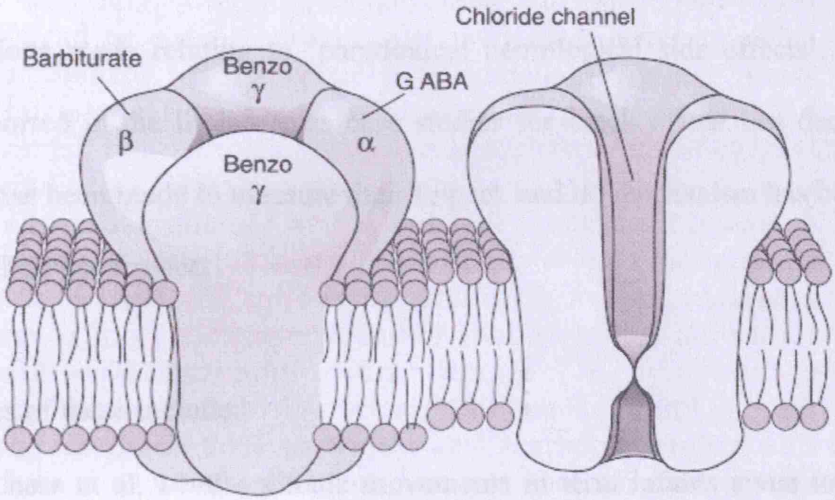

Gephyrin post-natally up-regulated
Chapter 2
KCC2 post-natally up-regulated
Chapter 2
Developmental change is activity dependent
Chapters 2&3
'Switch' in the nature of GABA/glycinergic transmission in vivo
Chapter 4
The change involves higher centres
Chapter 5
Clinical adverse events of benzodiazepine use in the youngest infants
Appendix 1

Appendix 1

GABAergic Excitation in Clinical Practice

A1.1 Introduction

This thesis has focussed on the postnatal regulation of functionally important proteins involved in synaptic inhibition (see chapters 2 & 4), and has confirmed the existence of a functional developmental 'switch' in GABAergic signalling in nociceptive processing *in vivo* (see chapter 3). Although the i*n vivo* work presented here deals exclusively with animal behavioural studies, there are likely to be important clinical consequences of these findings. Benzodiazepines are drugs used widely for seizure control, anxiolysis and sedation. Their mechanism of action is via a modulatory site on the $GABA_A$ receptor, where their presence increases the frequency of opening of the trans-membrane chloride channel, for a given amount of GABA.



In the mature nervous system, more chloride channel opening leads to greater chloride influx and enhanced inhibition. However, if the intracellular chloride concentration were high, as is the case in neonatal rat neurons, the increased opening of the channel would lead to increased chloride efflux and enhanced excitation. Side effects of this class of drugs in adult clinical practice are known to include drowsiness, respiratory depression, hypotension and amnesia (British National

Formulary, March 2005). These are all consistent with enhancement of GABAergic inhibition. It would appear possible that the adverse events of benzodiazepines in the youngest patients could be qualitatively different in the light of the data presented in this thesis.

Benzodiazepines are commonly used in neonatal practice for sedation during mechanical ventilation and for procedures. Their use has been surrounded by much controversy, as their effects in the neonatal population have indeed been found to be unpredictable (Ng et al, 2003). Although pharmacokinetics differences exist in the handling of benzodiazepines between neonates and older children, accounting for some of the differences seen (de Wildt et al, 2001), this would only be expected to produce a quantitative effect. It therefore does not account for some of the observations made relating to 'paradoxical neurological side effects'. These have been reported in the literature as case studies for much of the last decade, but no attempt has been made to measure their impact, and no mechanism has been found to explain their occurrence.

Examples of these include:

- Chess et al, 1998 – Clonic movements in term infants given lorazepam for sedation;
- Waisman et al, 1999 – Myoclonus in very low birthweight preterm infants given midazolam boluses;
- Zaw et al, 2001 – Flumazenil reverses myoclonic-like movements associated with midazolam;

- Montenegro et al, 2001 – Epileptic manifestations induced by midazolam in the neonatal period;
- Ng et al, 2002 (Review) – Seizures, myoclonus, changes in tone, and extra-pyramidal movements reported with benzodiazepine use in the newborn;
- Ter Horst et al, 2004 – Burst suppression on EEG may be induced by midazolam

In this final chapter, a postal questionnaire has been used to survey practicing UK neonatologists on the subject of benzodiazepine use within their clinical practice. The respondents were asked to comment on all adverse events, and were not informed of our hypothesis prior to completing the questionnaire, in an attempt to limit recall bias. It would follow that if the GABA 'switch' was responsible for these 'paradoxical' neurological effects, they may be reported more frequently among the younger infants. As the true frequency of these adverse events has not been investigated, these results will provide a useful guide to the true clinical picture.

A1.2 – Materials and Methods

A postal questionnaire was employed to gauge the importance of the various adverse events reported as a result of using the benzodiazepine, midazolam, in neonatal intensive care units (NICUs). All of the adverse events listed in the Cochrane systematic review (Ng et al, 2003) of the use of midazolam on NICU were listed, and neonatologists were asked to score their impact on a scale of 1-5, based on their own experience. The clinicians were asked to consider infants of 32 weeks post conceptional age (PCA) and under separately from those of over 32 weeks. Space was also given for respondents to add further comments relating to their experience of midazolam use.

The questionnaire was 'blinded' as to its actual purpose by including all the side effects discussed in the Cochrane review including the known (appearing on data sheet) dose dependent adverse events as well as 'paradoxical neurological events'. In view of this, the participants were offered the chance to receive feedback on the true purpose of the study and the results obtained, at a later date, by providing an Email address. The questionnaire was assessed, prior to its use, by the Great Ormond Street Hospital / Institute of Child Health ethics committee. The British Association of Perinatal Medicine database of members was used to identify an appropriate group of neonatal practitioners. All members who were Consultants, Specialist Registrars, or Staff Grades were invited to take part in the study. Clinicians were excluded if their work address was outside the UK, if they were in a locum post, or if they were not currently working on an NICU. Consequently, the final group consisted of 400 members.

A stamped addressed envelope was provided for returning the questionnaire. A number of questionnaires were excluded after their return because of being incorrectly completed, because the person was no longer working on the NICU targeted, or because the respondent was no longer working on an NICU responsible for the care of infants under 32 weeks post conceptual age.

A1.3 – Results

252 (63%) questionnaires were returned in the three-month period following their dispatch. Of these 218 (55%) had been correctly completed by an appropriate clinician, and were subsequently analysed.

A1.3.1 Paradoxical excitation and myoclonus occur with two thirds the frequency of established side effects (see appendix fig 1.1)

Myoclonus and paradoxical excitation were reported by a quarter of all respondents in the group aged 32 weeks PCA and under. Conventionally recognised side effects such as respiratory depression, and hypotension were found in 30-40 % of cases. This represents a previously undocumented, significant degree of neurological morbidity.

A1.3.2 All 'paradoxical neurological adverse effects' occur with a greater frequency in the youngest infants (see appendix fig 1.1 & 1.2)

All four of the 'paradoxical neurological adverse events' occurred with a greater frequency in the younger age group. This pattern was not consistent with the more 'conventional' side effects.

A1.3.3 In the youngest age group, seizures, myoclonus, and paradoxical excitation were as likely to be described as 'common' as respiratory depression or hypotension (see appendix fig 1.2)

The respondents reporting adverse events were divided into those reporting them as 'occasional' or 'common'. In the infants of 32 weeks PCA or less, seizures, myoclonus and paradoxical excitation were described as 'common' by as many

clinicians as were respiratory depression and hypotension. In the older babies, respiratory depression was clearly the side effect most likely to be described as 'common'.

A.1.3.4 When the score (1-5) is taken into account, the relative impact of 'paradoxical neurological adverse events' is of a similar order of magnitude to conversional side effects (see appendix fig 1.3)

The relative impact of myoclonus is three quarters that of respiratory depression in infants <32 weeks PCA, making it one of the most significant adverse events overall.

A1.3.5 The reported incidence of respiratory depression increases with age (see appendix fig 1.4)

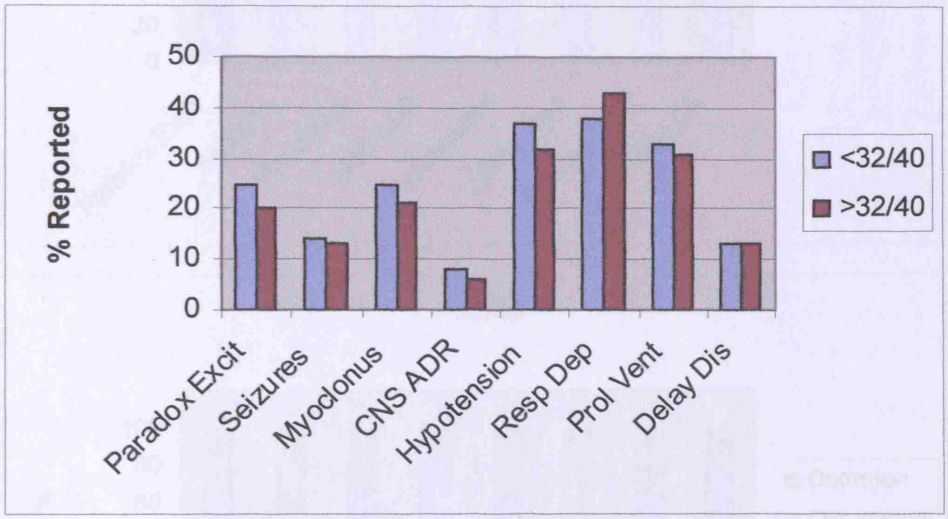
Respiratory depression is more likely to be reported as present and as 'common' with increasing age, being reported by almost half of all responders in the older babies, however this age difference does not reach statistical significance.

A1.3.6 Conversely, the reported incidence of myoclonus decreases with age (see appendix fig 1.4)

Myoclonus, on the other hand, is more likely to be reported in the younger age group, with a decrease in reported rates after 32 weeks PCA. Although this trend is shared by many of the 'paradoxical' neurological adverse events, the age differences do not attain statistical significance.

A1.3.7 Many responders gave unprompted examples of specific clinical situations involving neurological adverse events following midazolam use (See appendix fig 1.5)

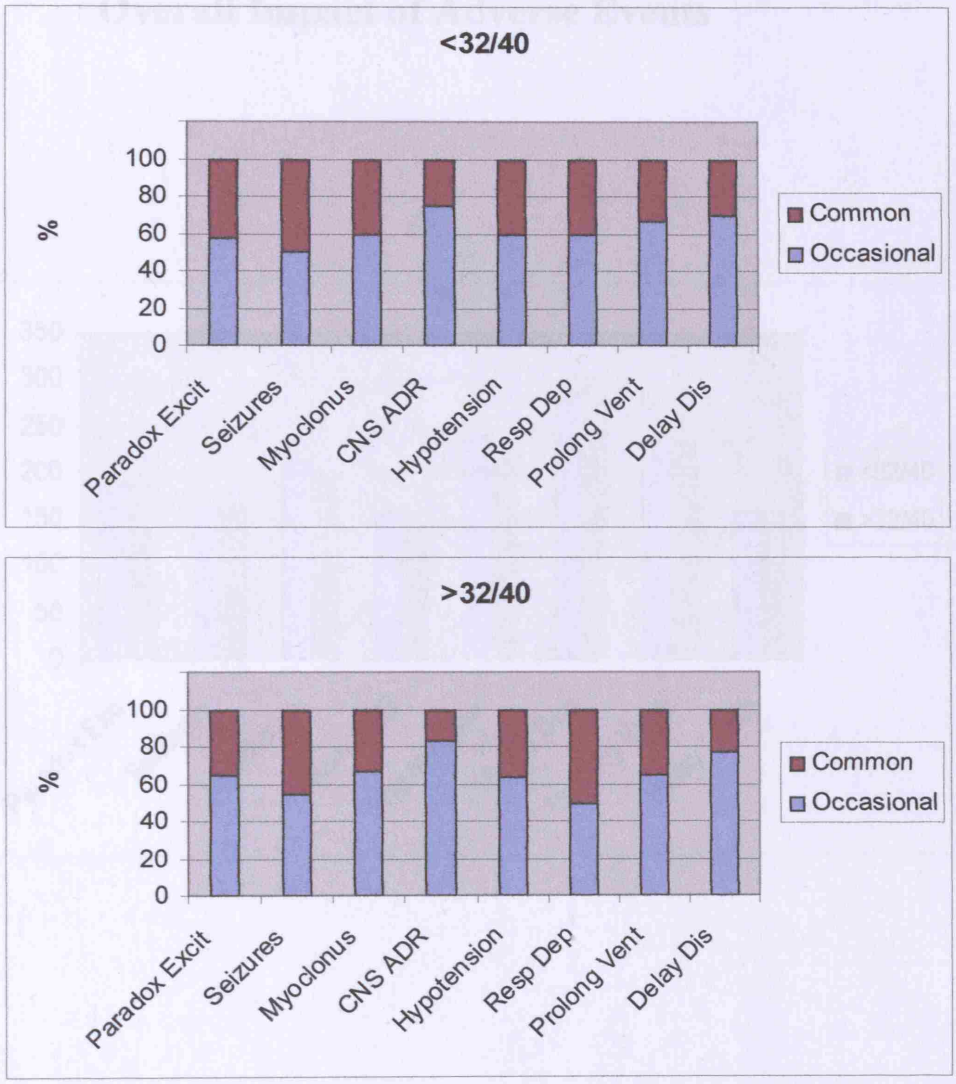
Reported Frequency of Adverse Events of Midazolam in NICU



Appendix Fig 1.1

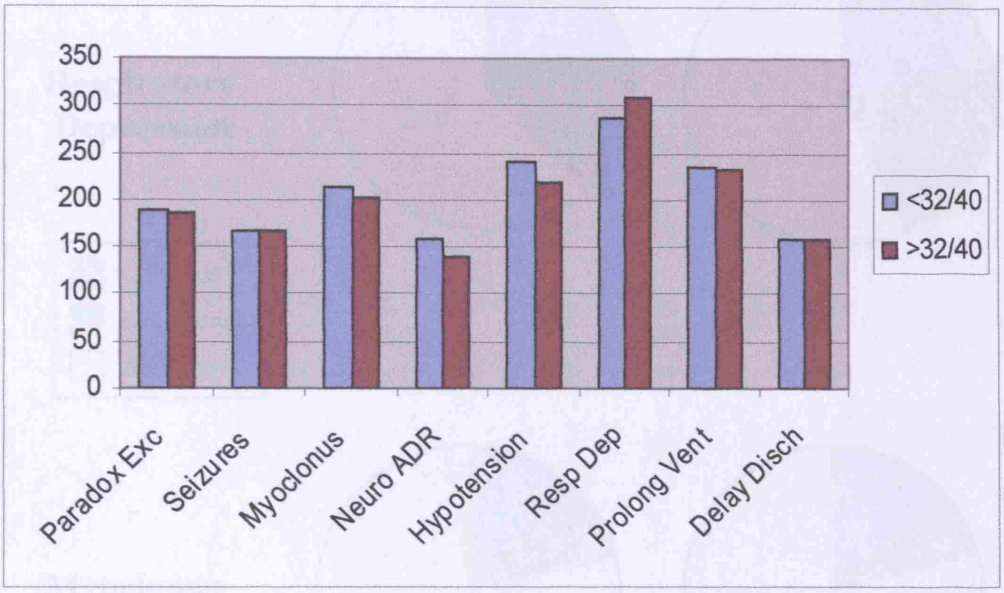
Reported frequency of adverse events following the use of midazolam on NICU, divided by age group (<32 weeks vs >32 weeks). Reporting rates for paradoxical excitation and myoclonus are high, being of comparable magnitude to dose dependent, recognised side effects. Both of these 'paradoxical' neurological side effects show a trend towards occurring with greater frequency in the youngest infants.

Frequency of Adverse Events Reported



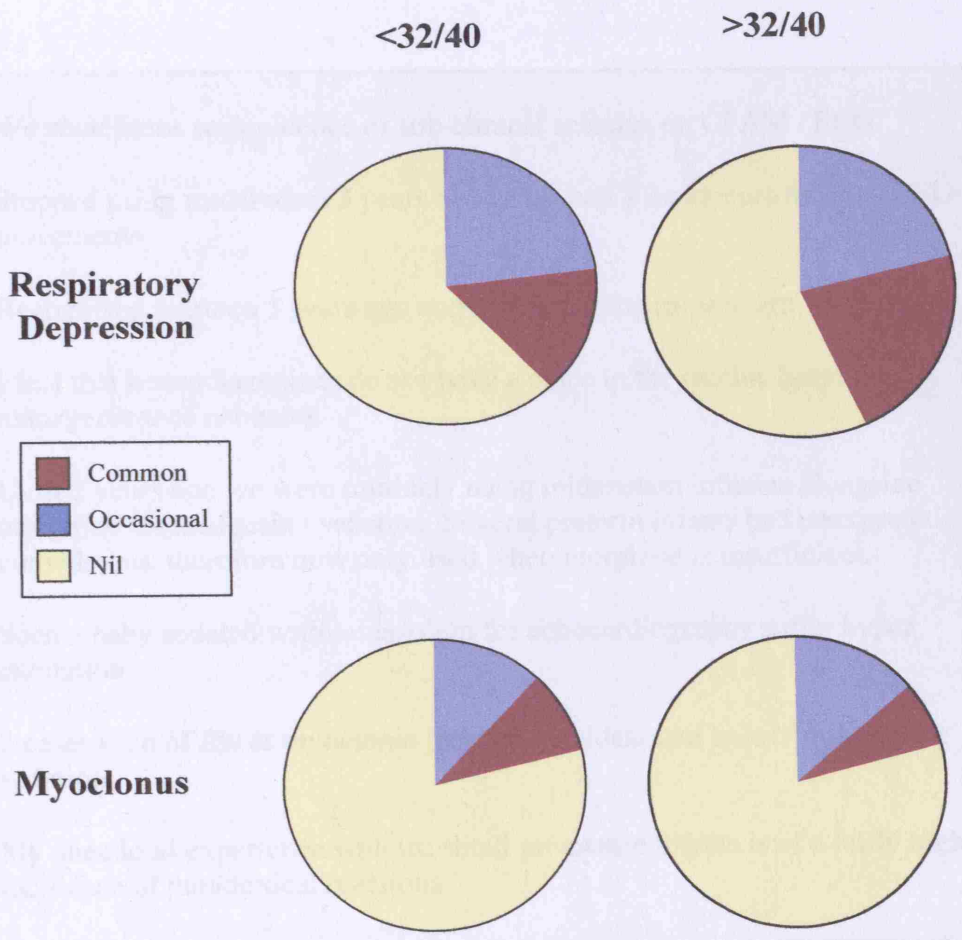
Appendix Fig 1.2
Relative frequency of the reported adverse events occurring with the use of midazolam on NICU. All reported 'paradoxical neurological adverse events' occur with greater frequency in the younger age group, although the difference does not attain statistical significance.

Overall Impact of Adverse Events



Appendix Fig 1.3
The relative impact of each adverse event is calculated by adding together the impact scores (0-5) for each report of an adverse event. The impact of myoclonus is found to be three quarters that of respiratory depression in the younger age group.

Developmental Progression of Midazolam Side Effects



Appendix Fig 1.4
Although the age related differences do not reach statistical significance, respiratory depression is reported with a greater frequency with increasing post conceptional age. Conversely, myoclonus appears to decrease frequency with maturity.

Anecdotal Comments From Questionnaire Replies

- We sometimes see evidence of sub-clinical seizures on CFAM / EEG
- Stopped using midazolam 3 years ago as we had 2 incidences of seizure-like movements
- Recognised seizures 5 years ago and stopped using midazolam since
- I feel that benzodiazepines do not have a place in the routine hospital management of neonates
- Until 2 years ago we were routinely using midazolam infusion alongside morphine for analgesia / sedation. Several preterm infants had unexpected convulsions, therefore now only used when morphine is insufficient.
- Seen a baby sedated with midazolam for echocardiography suffer hyper-excitation
- 2 cases seen of fits & myoclonic jerks after midazolam bolus / infusion for sedation
- My anecdotal experience with the small premature infants is of a fairly high incidence of paradoxical reactions

Appendix Fig 1.5
Selection of comments made by responding clinicians in the free text section

A1.4 – Discussion

Although, the questionnaire used in this study draws on the subjective recall of individual clinicians, it has revealed some interesting trends in the adverse events produced by midazolam in neonatal practice.

A1.4.1 Methodological considerations

63% of questionnaires were returned within a 3-month period, constituting a reasonable response rate for a postal questionnaire without follow up. Of these 55% were useable (correctly completed by appropriate clinicians), representing a large sample of UK neonatal units. Despite the questionnaire being effectively 'blinded' as to its purpose, the debate about neurological side effects of benzodiazepines in NICU is well publicised, and respondents may have guessed the purpose of the study. The decision was made to keep the questionnaire simple in order to maximise the response rate from busy clinicians. This means that questions related to subjective recall, rather than asking for individual patient details, thus avoiding the need for accessing medical notes. As such, reported rates of adverse events are only approximate, but provide a useful guide for further study.

A1.4.2 Neurological Adverse Events

Paradoxical excitation and myoclonic movements stand out as being reported very frequently. This is in keeping with the case reports published to date (Waisman et al, 1999; Chess et al, 1998; Zaw et al, 2002; Ng et al, 2002). Although the difference did not reach statistical significance, these were also reported with greatest frequency in the youngest infants, in agreement with the observations of Waisman et al, and in keeping with the animal data presented in this thesis. 32 weeks PCA corresponds

loosely to P10 in the infant rat (Fitzgerald, 1988), a developmental stage at which GABAergic excitation begins to shift to inhibition in several areas of the rodent CNS including the hippocampus, brainstem and cerebral cortex (Ben-Ari et al, 1989; Kullmann and Kandler, 2001; LoTurco et al, 1995). Therefore, it follows that human infants under 32 weeks PCA may suffer more neurological adverse events if these are related to GABAergic activity. The involvement of GABAergic signalling in the myoclonic movements produced by midazolam was confirmed by Zaw and colleagues, who administered the benzodiazepine-site competitive antagonist flumazenil to affected infants and found the effect to be reversed (Zaw et al, 2001). This inhibitory role for flumazenil is in accordance with the inhibitory behavioural effect of gabazine and bicuculline reported in chapter 3 of this thesis.

A1.4.3 Developmental Changes in Adverse Events Reported

Respiratory depression, which may be considered an inhibitory action of GABA at the level of the brainstem, shows a trend towards increasing with post-natal development. This would be consistent with the observations made relating to the developmental expression of KCC2 in the brainstem in chapter 3 of this thesis. KCC2 appears to be developmentally up regulated between the ages of P3 and P21, allowing GABA to behave in an increasingly inhibitory fashion. Conversely, myoclonus, which is likely to reflect an excitatory role of GABA (as demonstrated by the findings of Zaw et al), is reported to occur more frequently in the babies of 32 weeks PCA or less, showing a trend towards reduction with increasing maturity. Together these observations offer a putative mechanism for the unpredictable and counterintuitive side effects associated with the used of midazolam in neonatal practice.

A1.4.4 Conclusion

The Cochrane systematic review of midazolam use for sedation in the neonatal intensive care unit concluded that there were safety concerns associated with its use and that further research was needed on both its effectiveness and safety (Ng et al, 2003). This postal questionnaire based study has sought to quantify the safety concerns relating to its use in the UK. Furthermore, we have suggested a putative mechanism to explain some of the unexpected effects previously reported. However, more detailed, case-by-case reporting of adverse events is needed to further clarify the problem. At present, this relies on spontaneous reporting by individual clinicians, through the submission of 'yellow cards' to the Medicines and Healthcare Products Regulatory Agency (MHRA). In the current study, although two thirds of responders reported at least one adverse event, only 2% has submitted such a report to the relevant authorities, despite neonatal side effects being singled out by the MHRA as an area where reports were important. This reflects poorly on the system of spontaneous reporting, and suggests that a more rigid study method is needed.

A prospective clinical study in human infants would also be an interesting further area of work. Preliminary plans are underway for a study of mechanical withdrawal thresholds in human infants receiving a single dose of midazolam prior to closure of a patent ductus arteriosus, before and after the dose.

Publications

Publications

Hathway G, Harrop E, Baccei M, Walker S, Moss A & Fitzgerald M (2006) A Postnatal switch in GABAergic control of spinal cutaneous reflexes. Eur J Neurosci 23(1):112-8.

International Meetings

Poster presentation, International Society Paediatric Pain meeting , Sydney 2003: "The Developmental Expression of Gephyrin, a scaffolding protein at inhibitory synapses"

Poster presentation, European Society for Developmental and Paediatric Pharmacology meeting, Maarburg, 2004: " The Behavioural Effects of Intrathecal Injection of GABA, glycine and their Antagonists in Neonatal Rat Pups" – Winner of European Young Investigator award

Oral Presentation at European Paediatric Clinical Pharmacology Workshop, in July 2005; 'GABA Excitation and Midazolam use on NICU'

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Submitted for publication

'Normal Inhibitory Tone is Present in Neonatal Rat Spinal Cord Despite Low Levels of the KCl Cotransporter KCC2' Bremner L, Harrop JE, Baccei ML, Moss A & Fitzgerald M

Chapter 7

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